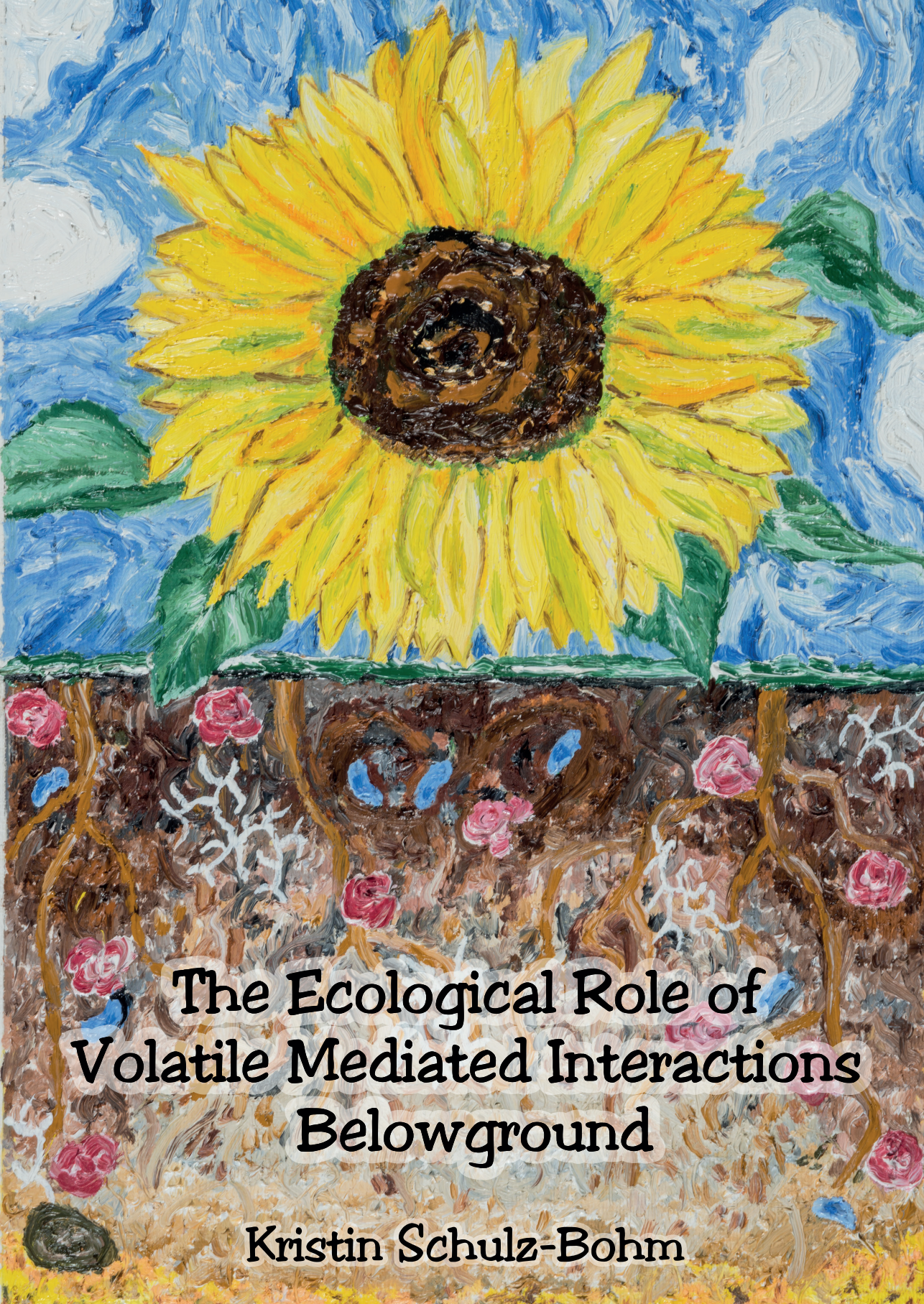

The Ecological Role of Volatile Mediated Interactions Belowground

Kristin Schulz-Bohm

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The Ecological Role of Volatile Mediated Interactions Belowground

Kristin Schulz-Bohm

Thesis

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"Look deep into nature,
and then you will understand everything better."

- A Monster Tree Harvester * -

* This is a wrongly assigned quote and intented to be a joke. Please laugh!
Original author: Albert Einstein.

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Abstract

In the porous network of soil, microbes are unevenly distributed. Interactions between soil (micro-) organisms that are physically separated could be mediated by volatile organic compounds (VOCs). VOCs are small, partially very smelly, molecules that can diffuse through air- and water-filled soil pores. Microbes, similar to plants, produce a diverse set of VOCs. The importance of these compounds in communication and competitiveness between microbes and plants in soil is increasingly recognized. However, our understanding on the relevance of VOCs-mediated interactions belowground is still limited. The aim of this thesis was to reveal novel insights into the ecological role of VOCs in microbial interactions and community dynamics in soil. A soil model system which more closely reflects conditions of soil environment in and around the rhizosphere was designed to study VOCs-mediated interactions between bacteria-bacteria, bacteria-fungi and plant-bacteria. Furthermore, novel aspects on the ecological role of VOCs in interactions of soil microorganisms such as protists were examined. Results of this thesis revealed that microbial interactions and shifts in the community composition strongly affect the volatile emission in soil. In this context, bacteria associated to the fungus can significantly influence the VOCs production and fitness of the fungal host. Moreover, within this thesis it was demonstrated that VOCs produced by microbes in the rhizosphere or plant roots can have a significant long distance effect on microorganisms in the surrounding nutrient-depleted bulk soil. For instance, VOCs released by bacterial interactions in the rhizosphere could stimulate the activity of distant starved bacteria. Furthermore, it was shown that plants can attract (beneficial) bacteria by root-VOCs. These results suggest that the rhizosphere effect might not be restricted to narrow zone – the few millimeters around the roots – but is further expanded by VOCs-mediated interactions. Interestingly, VOCs can also play a role as long-distance messenger in interactions between bacteria and protists. It was shown that bacterial VOCs such as terpenes affect protist activity and motility. This was mostly correlated to responses in direct feeding-interactions. Accordingly, bacterial VOCs could serve as signals for protists to find suitable prey. Overall, findings of this thesis provide novel information on the complexity of VOCs-mediated interactions in soil and contribute to our knowledge on the importance of VOCs-mediated (chemical) communication in ecosystem functioning belowground.

CHAPTER 1

General Introduction and Thesis Outline

The Habitat Soil - A Belowground Labyrinth with many Opportunities

Soil is not simply 'dirt', but a highly complex blend of weathered mineral rocks and organic material mixed with biota (Dighton 2003). Soil microbiota such as bacteria and fungi play a substantial role in the decomposition and mineralization of organic and inorganic material, plant nutrition, and influence the formation of soil aggregates (Vos *et al.* 2013).

Soil aggregation is a key component of soil structure that creates a three-dimensional network of water- and air-filled pores (Vos *et al.* 2013; Rillig *et al.* 2017). The availability of resources, redox-potential, pH and aeration can strongly vary between the pores and quickly change over time. Hence, the spatio-temporal heterogeneities in soil create many different niches for soil microbes resulting in an enormous biodiversity even at small scale in soil (Dechesne *et al.* 2007; Young *et al.* 2008). Moreover, frequent modifications in soil aggregation in combination with fluctuations of soil conditions such as water content, pH and temperature gradients have a strong impact on the evolutionary shaping of soil microbial communities (Crawford *et al.* 2005; Rillig *et al.* 2017). Generally, in terms of biodiversity, soil is one of the most important habitats of microbes. One gram of soil can easily exceed more than 10^9 bacterial cells or more than 10^5 different bacterial species (Gans *et al.* 2005; Roesch *et al.* 2007; Uroz *et al.* 2010). However, only 1 % of the soil surface is actually covered by microorganisms (Young *et al.* 2008).

Bacteria usually occupy micropores (< 10 µm) where they can escape from predation. As essential aquatic organisms, similarly as other soil inhabitants like protists and nematodes, bacteria live in thin water-films adhering on pore surfaces (Young *et al.* 2008). Therefore, those organisms are strongly reliant on the availability of water that connects air-filled pore space and allows them to develop and to explore a wider soil ecosystem (Figure 1.1). In contrast, filamentous fungi are able to bridge air-filled pores with their hyphae to explore new resources (Foster 1988). Bacteria can use those fungal hyphae as 'highway' to move within the porous soil network (Simon *et al.* 2015).

Most parts of soil are low in nutrient availability. Therefore, soil microbes accumulate at any suitable nutrient source that creates colonization hotspots but this also promotes strong interspecific competition. One of those microbial colonization hotspots in soil is the rhizosphere, the narrow zone surrounded and influenced by plant roots.

The Rhizosphere - An Oasis for Microbial Life Belowground

The rhizosphere is accounted as one of the most dynamic interfaces on earth (Philippot *et al.* 2013). Since large parts of soil are limited in nutrient access, the rhizosphere represents an oasis for soil microorganisms due to the release of rhizodeposits by plant roots including root exudates, border cells and mucilage. The rhizosphere is populated by a high number of many different bacteria, fungi and protozoa as well as invertebrates such as nematodes and arthropods (Bonkowski *et al.* 2009, Buée *et al.* 2009). Various intra- and inter-kingdom interactions of those rhizosphere inhabitants can be crucial for plant growth, health and disease suppression (Whipps 2001; Raaijmakers *et al.* 2009; Frey-Klett *et al.* 2011; Mendes *et al.* 2013; Philippot *et al.* 2013; Lareen *et al.* 2016; Jousset 2017). For example, quorum-sensing signals released by intra- and interspecific interactions of bacteria can induce plant systemic resistance and stimulate plant growth (Schuhegger *et al.* 2006; Müller *et al.* 2009). It was also shown that the presence of protozoa in the rhizosphere can stimulate the activity of plant-beneficial traits of bacteria (Weidner *et al.* 2017). Moreover, microbe-microbe interactions in the rhizosphere can trigger the suppression of soil-borne plant pathogens and, accordingly, promote plant growth (Garbeva *et al.* 2011a). For instance, interaction of the fungus *Fusarium oxysporum* with ectosymbiotic bacteria inhibits fungal virulence as well as induces antagonistic activity against phytopathogenic *Fusarium* (Minerdi *et al.* 2008; Minerdi *et al.* 2009). Similarly, pathogenicity of the fungus *Botrytis cinerea* can be reduced by interaction with soil bacteria that are able to degrade oxalic acid (Schoonbeek *et al.* 2007).

Besides the fact that the 'rhizobiome' (i.e. rhizosphere microbiome) affects the host plant, plants do also significantly influence the microbial community around the roots (Garbeva *et al.* 2007; Broeckling *et al.* 2008; Berg & Smalla 2009; Dennis *et al.* 2010; Mendes *et al.* 2013; Haichar *et al.* 2014; Sasse *et al.* 2017). For instance, some plant species release compounds such as flavonoids which can attract symbiotic nitrogen-fixing bacteria of *Bradyrhizobium japonicum* (Barbour *et al.* 1991), whereas other plant species excrete antimicrobial substances suppressing plant pathogens (Bais *et al.* 2006; Berg & Smalla 2009; Mendes *et al.* 2013; Rasmann & Turlings 2016; Van Dam & Bouwmeester 2016). In general, the rhizobiome is affected by several factors including physico-chemical conditions such as soil type, organic content, and soil moisture (Griffiths *et al.* 1999; De Ridder-Duine *et al.* 2005; Singh *et al.* 2007; Carson *et al.* 2010; Lareen *et al.* 2016;) as well as plant physiology (Chiarini *et al.* 1998; Paterson *et al.* 2007; Berg & Smalla 2009; Philippot *et al.* 2013), and plant genotype (Buée *et al.* 2009; Sasse *et al.* 2017).

Most members of the rhizobiome are part of a complex food web that utilizes the nutrients released by the plant (Mendes *et al.* 2013). It was long time assumed that bacteria,

because of their fast growth, are primarily consumers of easy degradable root exudates consistent of low molecular weight organic compounds such as sugars, amino and organic acids (De Boer *et al.* 2005). However, also saprotrophic (i.e. organotrophic) fungi abundant in the rhizosphere significantly contribute to the degradation of root-exudates (Treonis *et al.* 2004; Denef *et al.* 2007; Buée *et al.* 2009; Hannula *et al.* 2012). Hence, organotrophic bacteria and fungi must have developed adequate strategies to survive and thrive in the rhizosphere. For instance, to escape competition in the rhizosphere, some bacteria such as *Collimonas* created opportunities to explore new niches like the consumption of fungal-derived substrates (De Boer *et al.* 2004; Leveau *et al.* 2010).

To inhibit competing microbes, bacteria and fungi produce a large set of secondary metabolites including antibiotics, bacteriocins, biosurfactants, and toxins (Whipps 2001; Riley & Wertz 2002; Raaijmakers *et al.* 2009; Buée *et al.* 2009; Mendes *et al.* 2013; Tyc *et al.* 2017b). Some of those compounds such as antibiotics function in a concentration-dependent manner, acting as growth suppressor at high concentrations and at low concentrations as signaling compound involved in intra- and interspecific interactions (Raaijmakers & Mazzola 2012). Furthermore, most bacteria and fungi can produce more than one antibiotic compound with overlapping or different degrees of antimicrobial activity (Mendes *et al.* 2013). Besides growth-suppressive soluble secondary metabolites also volatile organic compounds, a group of small molecules of low molecular weight that have been long time overlooked, can play important roles in microbial interactions belowground.

Volatile Compounds - Small Molecules of Great Importance in Belowground Interactions

Research of the last decades revealed that, besides plants, soil microbes produce a large set of diverse inorganic and organic volatile compounds (Stotzky & Schenck 1976; Schulz *et al.* 2010; Effmert *et al.* 2012; Peñuelas *et al.* 2014; Schenkel *et al.* 2015; Lemfack *et al.* 2017). Some of those compounds can be quite odorous, such as the earthy smelling geosmin produced by *Streptomyces* or 1-octen-3-ol emitted by many fungi that can be perceived as the typical fungal smell (Lemfack *et al.* 2014; Schenkel *et al.* 2015).

Volatile organic compounds (VOCs) belong to different chemical classes. While bacterial VOCs are dominated by acids, alcohols, alkanes, alkenes, esters, ethers, ketones, lactones, pyrazines, sulfur compounds and terpenes, fungi produce mainly alcohols, aldehydes, alkenes, alkines, benzenoids, esters, ethers, thiofurans and terpenes (Effmert *et al.* 2012; Peñuelas *et al.* 2014; Schenkel *et al.* 2015; Lemfack *et al.* 2017). Also plants can

release a great variety of VOCs belowground. It is estimated that VOCs constitute about 1 % of plant secondary metabolites (Venturi & Keel 2016). The volatile blend released by plant roots consists of acids, aldehydes, alcohols, benzenoids, esters, ketones, terpenes, nitrogen and sulfur compounds as well as volatile phytohormones such as ethylene and methyl salicylate (Maffei *et al.* 2011; Dudareva *et al.* 2013; Peñuelas *et al.* 2014; Selvaraj 2015; Van Dam *et al.* 2016).

A majority of VOCs is produced as side-products of primary and secondary metabolism by biosynthetic pathways such as aerobic heterotrophic carbon metabolism, fermentation, amino acid degradation, terpenoid biosynthesis and sulfur reduction (Schulz & Dickschat 2007; Korpi *et al.* 2009; Dudareva *et al.* 2013; Peñuelas *et al.* 2014; Van Dam *et al.* 2016; Dickschat 2017). However, VOCs are not simply metabolic 'waste products' which is indicated by their diverse biological activities (Effmert *et al.* 2012; Schmidt *et al.* 2015; Van Dam *et al.* 2016). Furthermore, studies revealed that for bacteria the production of certain VOCs with antifungal activity including dimethyl sulfide is regulated by GacS/GacA two-component regulatory system or by quorum sensing (Han *et al.* 2006; Müller *et al.* 2009; Cheng *et al.* 2016; Ossowicki *et al.* 2017).

VOCs are characterized by a low molecular weight (< 300D), high vapor pressure (> 0.01 kPa at 20°C) and low boiling points enabling an evaporation at normal temperatures and pressure (Insam & Seewald 2010; Effmert *et al.* 2012; Bitas *et al.* 2013). Due to those physico-chemical characteristics, VOCs can easily diffuse through air- and water-filled soil pores with an effective range of potentially several centimeters (Hiltpold & Turlings 2008). Hence, VOCs can be ideal info-chemicals to mediate short- and long-distance intercellular and organismal interactions (Figure 1.1). In fact, several studies indicated that microbial as well as plant VOCs could play important roles in belowground intra- and interkingtom interactions where they can function as antimicrobials, carbon source, as well as info-chemicals affecting physiological and developmental processes (Wheatley 2002; Kai *et al.* 2009; Maffei *et al.* 2011; Effmert *et al.* 2012; Bitas *et al.* 2013; Peñuelas *et al.* 2014; Kanchiswamy *et al.* 2015a; Schmidt *et al.* 2015; Van Dam *et al.* 2016; Werner *et al.* 2016; Piechulla *et al.* 2017).

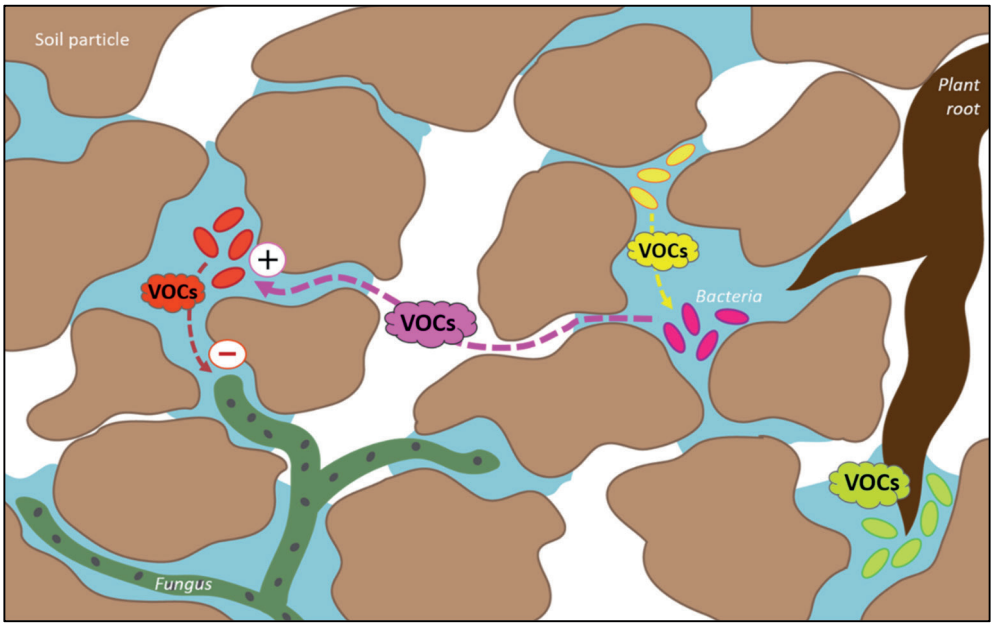

Figure 1.1. Volatile-mediated interactions belowground. In the porous soil matrix, bacteria live in water-filled pores while fungi can bridge air-filled pores with their hyphae. Communication (illustrated by plus sign) and competitive interactions (illustrated by minus sign) between physically separated soil organisms can be facilitated by volatile organic compounds (VOCs) that can diffuse through air- and water-filled soil pores. Microbial VOCs can play a role in intra- (e.g. bacteria-bacteria) and inter-kingdom interactions (e.g. bacteria-fungi or bacteria-plant).

The role of VOCs in microbe-microbe interactions

While VOCs-mediated interactions of plants were widely studied in the past (Maffei *et al.* 2011), the potential of microbes to produce a wide set of biologically active volatiles and their ecological impact in belowground interactions was largely ignored. Microbial VOCs (mVOCs) can play important roles in competitive interactions. For several bacteria and fungi, a production of VOCs with antibacterial and/ or antifungal activity was reported (Kai *et al.* 2009; Insam & Seewald 2010; Garbeva *et al.* 2011a; Leeder *et al.* 2011; Effmert *et al.* 2012; Bitas *et al.* 2013; Schmidt *et al.* 2015; Kanchiswamy *et al.* 2015a; Li *et al.* 2016; Van Dam *et al.* 2016; Werner *et al.* 2016; Piechulla *et al.* 2017). Especially soil microbes producing VOCs that inhibit plant pathogens are of major interest for sustainable crop protection (Fernando *et al.* 2005; Weisskopf 2013; Kanchiswamy *et al.* 2015a; Kanchiswamy *et al.* 2015b; Bailly & Weisskopf 2017). However, the sensitivity to VOCs can strongly vary, depending on individual bacteria-fungus or fungus-fungus interactions (Humphris *et al.* 2002; Wheatley 2002; Vespermann *et al.* 2007; Kai *et al.* 2008; Garbeva *et al.* 2014b; Schmidt *et al.* 2015; Werner *et al.* 2016;).

Besides the fungicidal effect, bacterial VOCs can also stimulate fungal growth (Vespermann *et al.* 2007; Briard *et al.* 2016). Furthermore, fungal morphology, enzyme activity and gene expression can be significantly influenced by VOCs of bacteria (Fiddaman & Rossall 1993; Wheatley 2002; Barbieri *et al.* 2005; Chaurasia *et al.* 2005; Minerdi *et al.* 2008; Zhao et al. 2011; Piechulla *et al.* 2017). For example, it was shown that bacterial VOCs could strongly affect the activity of laccase and tyrosinase (Mackie & Wheatley 1999; Crowe & Olsson 2001). Moreover, ammonia emitted by bacteria was shown to stimulate trap formation in nematode-trapping fungi (Wang *et al.* 2014; Su *et al.* 2016), demonstrating the role of mVOCs in tri-trophic interactions.

Ammonia can also act as signal molecule in bacteria-bacteria interactions and enhance, for example, antibiotic resistance as well as influence biofilm formation and pigmentation (Nijland & Burgess 2010; Bernier *et al.* 2011). Another group of well-known bacterial signal molecules are indoles. Studies revealed that indoles can affect spore formation, antibiotic resistance, virulence, biofilm formation and motility of the bacterial recipient (Lee *et al.* 2015). Besides the stimulation of antibiotic resistance (Audrain *et al.* 2015b), growth and the production of secondary metabolites including antibiotics can be induced by certain VOCs-mediated interactions between bacteria (Garbeva *et al.* 2014a; Schmidt *et al.* 2015).

In addition to inter-specific interactions, mVOCs can play an important role in intra-specific interactions of bacteria. For example, *Streptomyces venezuela* emit trimethylamine under nutrient-limitation to induce exploratory growth in distant cell members (Jones *et al.* 2017).

Intra-specific communication of bacteria is often mediated by quorum sensing (QS). Disruption of the QS signaling cascade, called quorum quenching (QQ), is an important mechanism during competition of bacteria (Chernin *et al.* 2011). Several studies revealed that VOCs have the potential to function as QS signal, QS regulator and QQ molecules (Schulz *et al.* 2010; Selvaraj 2015). For instance, ketones produced by bacteria including 2-nonanone, 2-heptanone and 2-undecanone can modulate quorum-sensing responses (Plyuta *et al.* 2014). Likewise Chernin *et al.* (2011) showed that the sulfur compound dimethyl disulfide emitted by *Pseudomonas fluorescens* or *Serratia plymuthica* acted as QQ molecule and suppressed the production of QS signal molecules of several bacteria. Dimethyl disulfide was also reported to exhibit strong antifungal activity and to stimulate bacterial growth (Kai *et al.* 2009; Garbeva *et al.* 2014a). Furthermore, the same compound can play an important role in microbe-plant interactions by improving plant growth and inducing systemic resistance (Huang *et al.* 2012a; Meldau *et al.* 2013). This shows that one single volatile compound can function in different ways depending on the interacting partner.

VOCs-mediated plant-microbe interactions

Bacteria and fungi produce a wealth of VOCs that can promote or inhibit plant growth as well as induce systemic resistance (Kai *et al.* 2009; Bailly & Weisskopf 2012; Bitas *et al.* 2013; Peñuelas *et al.* 2014; Kanchiswamy *et al.* 2015a; Selvaraj 2015; Li *et al.* 2016; Van Dam *et al.* 2016; Werner *et al.* 2016; Piechulla *et al.* 2017; Riedlmeier *et al.* 2017). A prominent bacterial volatile compound that stimulates plant growth and systemic resistance is 2,3-butanediol, specifically the isomer (2R,3R)-2,3-butandiol (Ryu *et al.* 2003; Ryu *et al.* 2004; Han *et al.* 2006; Chung *et al.* 2016). Besides the induction of resistance against plant pathogens, mVOCs can also promote tolerance of plants to abiotic stress such as salt or drought (Cho *et al.* 2008, Zhang *et al.* 2008). Generally, the induction of systemic resistance, stimulation of plant growth and increase in drought tolerance by exposure to mVOCs seemed to be mediated through modulation of hormone pathways in plant tissue (Ryu *et al.* 2004; Zhang *et al.* 2007; Cho *et al.* 2008; Bailly *et al.* 2014; Naznin *et al.* 2014; Bitas *et al.* 2015; Liu & Zhang 2015). On the other hand, plant growth can be enhanced due to the improvement of nutrient access by released VOCs of bacteria in the rhizosphere (Zhang *et al.* 2009; Meldau *et al.* 2013; Liu & Zhang 2015).

Interestingly, recent studies revealed that also phytopathogenic microbes can promote plant growth via VOCs (Bitas *et al.* 2015; Cordovez *et al.* 2017). The stimulation of lateral root growth, thereby, might be a very helpful strategy to increase the area for fungal colonization and infection. Similar modulations of root development were also shown for VOCs including sesquiterpenes released by beneficial ectomycorrhizal fungi (Ditengou *et al.* 2015), suggesting that certain fungal VOCs may be evolutionary conserved signal molecules facilitating infection of plant roots.

Plants release a great variety of VOCs that can function in multiple ways and significantly affect the surrounding soil organisms (Van Dam *et al.* 2016). Plant VOCs are mainly associated to negative effect of microbial growth and development (Wenke *et al.* 2010). However, VOCs released by roots including terpenes can also serve as carbon source (Kleinheinz *et al.* 1999; Owen *et al.* 2007; Del Giudice *et al.* 2008) or function as info-chemicals in plant-microbe interactions. For example, Ahmad *et al.* (2015) revealed that plant VOCs such as carvone, limonene, and borneol stimulated bacterial QS while α-terpineol and cis-3-nonen-1-ol inhibited bacterial QS.

Concluding remarks

Despite the increasing number of studies, our understanding on the ecological role of VOCs in microbial interactions belowground is still in its infancy. So far, about 1850 mVOCs of 945 different microbial species (i.e. fungi and bacteria) are listed in the mVOC database (Lemfack *et al.* 2017). However, one gram of soil can contain more than 10^5 different bacterial species (Gans *et al.* 2005). This shows that only a very small proportion of the complexity of microbial volatiles and its function is known. Specifically soil microbes such as archaea or protists are completely understudied in this regard. Furthermore, our current knowledge on the role of VOCs-mediated interactions belowground are mainly based on experiments under artificial culture conditions including nutrient-rich agar media that significantly affect the production of VOCs and can lead to opposing effects (Wenke *et al.* 2010; Blom *et al.* 2011; Garbeva *et al.* 2014b). Hence, new experimental systems closer to *in-situ* conditions in soil in combination with advancing technologies will help to gain a better understanding of the actual ecological role of VOCs in soil ecosystems.

Thesis Outline

Literature of the last decades suggests that VOCs play an important role in the communication and competitiveness between physically separated microorganisms in soil (Schmidt *et al.* 2015; Van Dam *et al.* 2016). However, our knowledge on the complexity and distribution of VOCs-mediated interactions in soil is still limited. The main aim of this thesis was to reveal new insights into the ecological relevance of VOCs in microbial interactions and community dynamics belowground. In this context, interactions between bacteria-bacteria, bacteria-fungi, bacteria-protists as well as plant-bacteria were examined (Figure 1.2).

Current knowledge on the role of microbial volatiles in belowground interactions was summarized in Chapter 2.

While many studies on the function of microbial volatiles in soil, described in Chapter 2, used artificial incubation conditions including one-to-one interactions, a soil model system that more closely reflects the *in situ* conditions in the heterogeneous soil environment along the rhizosphere was applied in Chapter 3 to study VOCs-mediated interactions between bacteria. We assessed how microbial interactions and shifts in the community composition in the rhizosphere affect the volatile emission and if these VOCs can stimulate the activity of starved bacteria in the surrounding nutrient-depleted bulk soil.

In Chapter 4, the role of VOCs in interactions between bacteria and protists was explored. Protists are major predators of bacteria but reaching the prey can be very costly. Thus, VOCs released by bacteria could give an early information on suitable prey located in distant soil pores. VOCs-mediated and direct interaction assays of different bacteria and protists were performed to investigate the relationship between stimulation of protists activity by bacterial VOCs and feeding preferences.

Besides protists also fungi are abundant in the rhizosphere and can affect microbial community dynamics (De Boer *et al.* 2015). Mutualistic and competitive interactions between bacteria and saprotrophic fungi were studied in Chapter 5 and 6. In Chapter 5, the impact of bacteria associated to the fungus *Mucor hiemalis* on the fungal fitness and behavior, including the production of VOCs of different biological activity, was studied. Novel molecular and microbiological approaches were applied to detect and isolate the fungus-associated bacteria.

In Chapter 6, it was aimed to reveal bacterial strategies (e.g. production of distinct VOCs) that lead to different competitive strength in fungal presence. Soil microcosms containing a synthetic bacterial community in presence or absence of the fungus *Fusarium culmorum* were set up and quantitative PCR in combination with transcriptomics and volatilomics was performed to link changes in bacterial abundance to potential mechanisms.

Besides microbe-microbe-interactions, also plants can affect microbial communities by released VOCs into the rhizosphere. However, the role of plant VOCs in the attraction of beneficial microorganisms is not known so far. In Chapter 7, an olfactometer system was designed to test the attraction of soil bacteria to VOCs emitted by *Carex arenaria* roots. In addition, it was examined if infection of *C. arenaria* by the facultative phytopathogen *F. culmorum* affects the VOCs profile and bacterial attraction. The same system was used to study the diffusion distance of various pure volatile compounds that were representative for the VOCs blend of *C. arenaria* roots and *F. culmorum*.

In Chapter 8, main findings of the thesis were summarized and discussed in the context of the role of VOCs-mediated interactions in ecosystem functioning. Furthermore, an outlook for future research directions on VOCs-mediated interactions belowground and potential applications of microbial and plant VOCs was given.

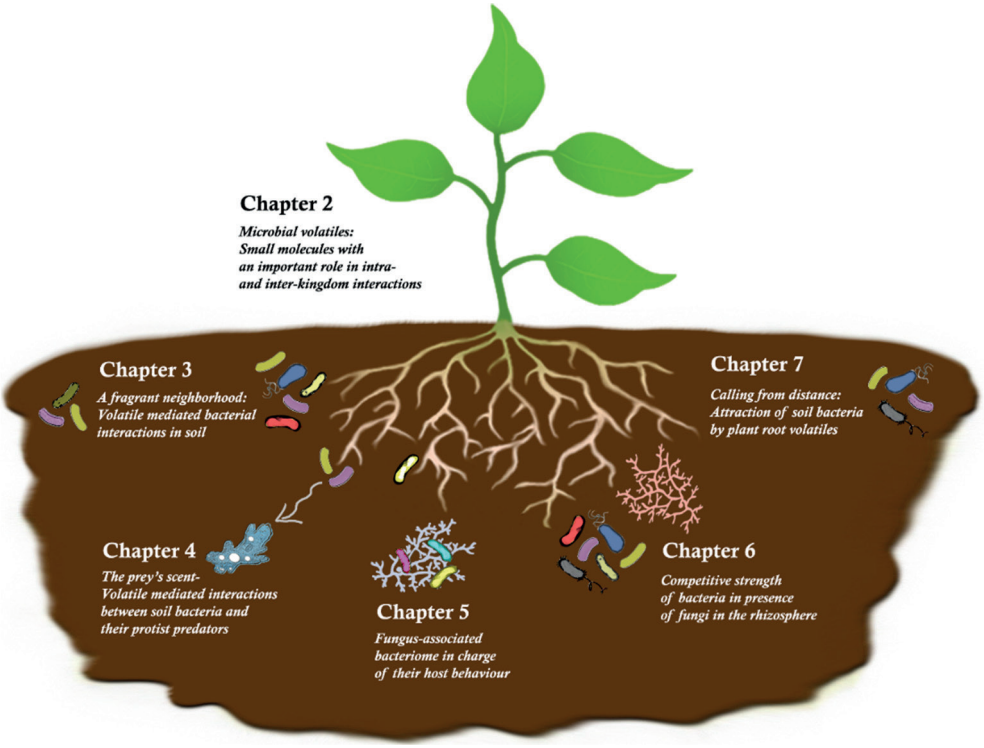

Figure 1.2. Thesis outline.

CHAPTER 2

Microbial Volatiles: Small Molecules with an Important Role in Intra- and Inter-Kingdom Interactions

Kristin Schulz-Bohm*, Lara Martin-Sanchez*, and Paolina Garbeva

* *Authors contributed equally to this work*

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Abstract

During the last decades research on the function of volatile organic compounds focused primarily on the interactions between plants and insects. However, microorganisms can also release a plethora of volatiles and it appears that microbial volatile organic compounds (mVOCs) can play an important role in intra- and inter-kingdom interactions. So far, most studies are focused on aboveground volatile-mediated interactions and much less information is available about the function of volatiles belowground. This minireview summarizes the current knowledge on the biological functions of mVOCs with the focus on mVOCs-mediated interactions belowground. We pinpointed mVOCs involved in microbe-microbe and microbe-plant interactions, and highlighted the ecological importance of microbial terpenes as a largely underexplored group of mVOCs. We indicated challenges in studying belowground mVOCs-mediated interactions and opportunities for further studies and practical applications.

Keywords:

volatile organic compounds, microbial interactions, bacteria, fungi, protists, plant-microbe interactions

Introduction

Many secondary metabolites have been reported to be involved in microbial interactions. One group of secondary metabolites produced by soil and plant-associated microorganisms, but largely unexplored to date, are the volatile organic compounds (VOCs). VOCs are typically small, odorous compounds (<C15) with low molecular mass (<300 Da), high vapor pressure, low boiling point, and a lipophilic moiety. These properties facilitate evaporation and diffusion aboveground and belowground through gas- and water- filled pores in soil and rhizosphere environments (Vespermann *et al.* 2007; Insam & Seewald 2010; Effmert *et al.* 2012). Microbial volatile organic compounds (mVOCs) belong to different chemical classes including alkenes, alcohols, ketones, benzenoids, pyrazines, sulfides, and terpenes (Schulz & Dickschat 2007; Lemfack *et al.* 2014; Lemfack *et al.* 2017; Kanchiswamy *et al.* 2015a; Schmidt *et al.* 2015). A recent meta-analysis by Schenkel *et al.* (2015) provided a comprehensive overview of VOCs derived from soil-borne microbes.

The production of mVOCs in soil is influenced by various factors including the growth stage of the microbes, nutrient availability, temperature, oxygen availability, pH, and soil moisture content (Wheatley 2002; Insam & Seewald 2010). Several recent studies reported that the production of certain mVOCs can be induced or suppressed during inter-specific microbial interactions (Garbeva *et al.* 2014a; Schulz-Bohm *et al.* 2015; Tyc *et al.* 2015; Piechulla *et al.* 2017).mVOCs were often considered to be by-products of primary metabolism, but recent findings revealed that many mVOCs demonstrate biological activity (Schmidt *et al.* 2015; Tyc *et al.* 2017a). Furthermore, in bacteria, the production of certain mVOCs is dependent on the GacS/GacA two-component regulatory system (Cheng *et al.* 2016; Ossowicki *et al.* 2017). These findings clearly disagree with the opinion that mVOCs are just waste products.

While soluble metabolites are often responsible for short distance interactions, VOCs are considered to be long-distance messengers (Tyc *et al.* 2017b; Westhoff *et al.* 2017). There are many types of microbial interactions occurring belowground such as bacteria–bacteria, fungi-fungi, fungi-bacteria, bacteria-protists, fungi-plant, bacteria-plant, and bacteria-fungi-plant interactions. However, most studies addressing belowground VOCs-mediated interactions are focused mainly on the root-emitted volatiles (recently reviewed by Delory *et al.* 2016).

The knowledge we have gained from research conducted over the last few years reveals that mVOCs can have both beneficial and harmful effects on other organisms (Effmert *et al.* 2012; Schmidt *et al.* 2015). mVOCs can provide organisms with rapid and precise ways to recognize neighboring organisms (both friends and foe) and to launch proper responses.

The aim of this review is to summarize the current knowledge concerning the role of mVOCs in intra- and inter-kingdom interactions, to pinpoint mVOCs (e.g., terpenes) involved in microbe–microbe and microbe–plant interactions as well as to indicate challenges in studying belowground mVOCs-mediated interactions and opportunities for further studies and practical applications.

VOCs in Microbe–Microbe Interaction

Bacteria-bacteria

Bacterial VOCs can have direct antagonistic effects against other bacteria. For instance, the sesquiterpene albaflavenone produced by *Streptomyces albidoflavus* revealed activity against *Bacillus subtilis* (Gürtler *et al.* 1994) and the emission of dimethyl disulphide by two rhizospheric bacteria, *Pseudomonas fluorescens* and *Serratia plymuthica*, showed bacteriostatic effects against two plant bacterial pathogens *Agrobacterium tumefaciens* and *Agrobacterium vitis* (Dandurishvili *et al.* 2011). *Pseudomonas fluorescens* WR-1 produces volatiles such as benzothiazole and 1-methyl naphthalene with bacteriostatic effects against the tomato pathogen *Ralstonia solanacearum* (Raza *et al.* 2016a). In fact, many species of *Pseudomonas* and *Bacillus* that are used as biocontrol agents against plant pathogens, have been reported to produce VOCs with antibacterial activity (Raza *et al.* 2016a; Raza *et al.* 2016b; Raza *et al.* 2016c; Xie *et al.* 2016; Rajer *et al.* 2017; Tahir *et al.* 2017a; Tahir *et al.* 2017b). For instance, a recent study revealed that VOCs produced by *Bacillus* spp., including benzaldehyde, 1,2-benzisothiazol-3(2 H)-one and 1,3-butadiene, had strong inhibitory activity against *R. solanacearum*, the causal agent of bacterial wilt disease (Tahir *et al.* 2017b). The mVOCs altered the transcriptional expression levels of several genes involved in motility and pathogenicity (e.g., global virulence regulator *PhcA*, type III secretion system, and extracellular polysaccharide [EPS] production) and induced systemic resistance by plants, which resulted in a decrease of wilt disease.

Several reports describe the effect of VOCs in bacterial virulence. For instance, 2,3 butanediol and acetoin are required for full virulence in *Pectobacterium carotovorum*. The same compounds can increase the production of virulence factors in *Pseudomonas aeruginosa* (Audrain *et al.* 2015a).

In contrast, VOCs produced by some bacteria can also have positive effects on the growth of other neighboring bacteria in the rhizosphere. For instance, VOCs from *Collimonas pratensis* and *S. plymuthica* are able to induce the growth of *P. fluorescens* Pf0-1 (Garbeva *et al.* 2014a. These VOCs induced expression of genes involved in motility in *P.*

fluorescens Pf0-1 and provoked an increase in the production of secondary metabolites with antibacterial activity against *Bacillus* (Garbeva *et al.* 2014a). This suggests that *C. pratensis* and *S. plymuthica* may be attracting and promoting the growth of *P. fluorescens* in a collaborative attempt to increase their chances against different bacterial competitors or soil fungal pathogens. Another example of the growth-promoting effect of VOCs was reported recently by Schulz-Bohm *et al.* (2015) which showed that VOCs released by mixtures of root exudate-consuming bacteria stimulated the activity and growth of distant nutrient-limited bacteria.

In addition to exerting antagonistic effects toward other bacteria, VOCs can also modify the behavior of other bacteria and modulate their resistance to antibiotics. Bacterial volatiles such as ammonia, trimethylamine, hydrogen sulfide, nitric oxide, and 2-amino-acetophenone can alter biofilm formation or dispersal or affect motility of bacteria (Audrain *et al.* 2015a; Raza *et al.* 2016a). Bacteria often make use of their motility to move to other areas with more resources and/or less competitors. In *Streptomyces venezuelae*, a new mode of development, so-called exploration, has been recently discovered that allows non-motile bacteria to access regions with more nutrients (Jones *et al.* 2017). *S. venezuelae* is able to produce hydrophilic fast growing non-branching vegetative hyphae, triggered by glucose depletion and a rise in pH, to presumably escape from poor nutrient areas. Interestingly, explorer cells can release signals for long distance communication with other members of the species to induce their exploratory growth. One of these signals is trimethylamine, which works not only as a signal to communicate with distantly located *Streptomyces* and induce exploratory growth but also displays antibacterial activity against *B. subtilis* and *Micrococcus luteus*, probably by rising the pH of the medium (Jones *et al.* 2017).

Fungi-bacteria

Fungal VOCs can play an important role in long distance fungal–bacterial interactions and can lead to different phenotypical responses in the interacting partners. For example, VOCs emitted by *Trichoderma atroviride* increased the expression of a biocontrol gene (*phlA*) in *P. fluorescens* that encodes the biosynthesis of 2,4-diacetylphloroglucinol (Lutz *et al.* 2004). A few recent studies demonstrated that the growth of some bacterial species can be suppressed by fungal VOCs (Werner *et al.* 2016) such as the VOCs that exhibit inhibitory effects on *B. cereus* and *B. subtilis* produced by the oyster mushroom *Pleurotus ostreatus* (Pauliuc & Dorica 2013).

Recently, Schmidt *et al.* (2016) screened the phenotypic responses of soil bacterial strains to volatiles emitted by several fungal and oomycetal soil strains under different nutrient conditions during different growth stages. Out of the phenotypical responses

tested such as growth alteration, antimicrobial activity, biofilm formation or motility, motility of bacteria (both swimming and swarming) was significantly positively or negatively affected by fungal and oomycetal VOCs. This finding could, therefore, reflect a potential strategy employed by the fungus to attract mutualistic bacteria toward itself and to repel competitors by manipulating their motility through the use of VOCs (Piechulla *et al.* 2017). Transcriptomics and proteomics analyses of *S. plymuthica* PRI-2C exposed to VOCs emitted by the fungal pathogen Fusarium culmorum, showed that *S. plymuthica* PRI-2C responded to the fungal VOCs with changes in gene and protein expression related to motility, signal transduction, energy metabolism, cell envelope biogenesis, and secondary metabolite production (Schmidt *et al.* 2017). The metabolomic analyses of *S. plymuthica* PRI-2C exposed to the fungal VOCs, the gene cluster comparison, and the heterologous co-expression of a terpene synthase and a methyltransferase revealed the production of the unusual terpene named sodorifen (Kai *et al.* 2010; Von Reuss *et al.* 2010) in response to fungal VOCs. These findings support the suggested importance of VOCs (and in particular terpenes) as signaling molecules in fungal–bacterial interactions.

Many soil bacteria can produce VOCs with antifungal effects and thus contribute to the phenomenon known as soil fungistasis where fungal propagules are restricted in their ability to grow or germinate (Garbeva *et al.* 2011a). Recently, Cordovez *et al.* (2015) revealed that VOCs produced by *Streptomyces spp*. exhibit antifungal properties against *Rhizoctonia solani* and may contribute to plant disease suppressiveness. Ossowicki *et al.* (2017) showed that VOCs from the tomato rhizosphere isolate *Pseudomonas donghuensis* P482 have strong antifungal and anti-oomycete activity which suggests that the antagonistic capabilities of this strain against plant pathogens are due to their volatile potential (Ossowicki *et al.* 2017). This effect of bacterial VOCs against oomycetes is not an isolated case and other *Pseudomonas* strains have been reported to have anti-oomycete activities (De Vrieze *et al.* 2015; Hunziker *et al.* 2015). In a recent report, VOCs produced by several *Lysobacter* strains growing in a protein-rich medium showed anti-oomycete activity whereas non-antagonistic VOCs were produced by these strains when grown on a sugar-rich medium. This indicates that the production of volatiles is highly dependent on growth conditions and nutrient availability (Lazazzara *et al.* 2017).

Fungi-fungi

The 1-octen-3-ol, one of the most prominent fungal VOC, known as the mushroom smell, is produced by a wide range of filamentous fungi and can function as a development signal among fungi (Miyamoto *et al.* 2014). The same compound was described to function in *Penicillium paneum* as a self-inhibitor signal in spore germination (Chitarra *et al.* 2004).

As developmental signals during population establishment, certain fungal VOCs act in a concentration-dependent manner to regulate conspecific mycelial growth and spore germination (Nemčovič *et al.* 2008; Stoppacher *et al.* 2010).

Fungal VOCs can have inhibitory effects and drive antagonistic interactions among fungi. For example, the endophytic fungi *Muscodor albus* and *Oxyporus latemarginatus* can strongly inhibit the growth of several plant pathogenic fungi, including *Botrytis cinerea* and *Rhizoctonia solani* (Strobel *et al.* 2001). VOCs emitted by *Trichoderma* spp. have a strong effect against plant pathogenic fungi such as *Fusarium oxysporum, Rhizoctonia solani, Sclerotium rolfsii, Sclerotinia sclerotiorum*, and *Alternaria brassicicola* (Amin *et al.* 2010). Similarly, VOCs such as 5-hexenoic acid, limonene, octanoic acid and 3,4-2H-dihydropyran produced by the non-pathogenic fungus *F. oxysporum* CanR-46 could inhibit mycelial growth of 14 fungal species including the pathogenic *Verticillium dahlia* (Zhang *et al.* 2015). Recently, a proteomic study demonstrated that fungal VOCs can interfere with essential metabolic pathways to prevent fungal growth (Fialho *et al.* 2016).

Some fungal species can detoxify the antifungal compounds produced by their microbial competitors. For example, *Fusarium graminearum* can detoxify the toxic compound 6-pentyl-alpha-pyrone, emitted by *Trichoderma harzianum* (Cooney *et al.* 2001). Fungal VOCs can be important carbon sources for fungi colonizing carbon-limited environments (Cale *et al.* 2016). Conversely, for fungi colonizing a more carbon-rich environment, VOCs may act, in a concentration-dependent manner, as semio-chemicals to mediate antagonistic and beneficial interactions between fungi.

Protists-bacteria

A very diverse and abundant group of soil microorganisms are protists (Protozoa) (Fierer & Jackson 2006; Geisen *et al.* 2015). Due to their grazing activities, protists play an important role in the soil food web and significantly affect carbon allocation and nutrient-cycling in the soil-plant-interphase (Geisen 2016). Most soil protists are known to be key predators of bacteria and can shape bacterial communities by selective feeding (Griffiths *et al.* 1999; Bonkowski & Brandt 2002; Rosenberg *et al.* 2009; Glücksman *et al.* 2010). Reaching suitable prey is very energy consuming (Jousset 2012). Thus, sensing their prey over long distances in the porous soil matrix would be very beneficial for protists. A recent study by Schulz-Bohm *et al.* (2017a) revealed that volatile organic compounds can play a key role in long-distance bacterial–protists interactions. By testing various volatile-mediated interactions between phylogenetically different soil bacteria and protists and comparing those with direct trophic interactions, they demonstrated that specific bacterial volatiles can provide early information about suitable prey. In particular, it was shown that terpenes such

as β-Linalool, β-Pinene, Germacrene D-4-ol or δ-Cadinene produced by *C. pratensis* Ter91 (Song *et al.* 2015b) can stimulate protist activity and motility suggesting that terpenes can be key components in VOCs-mediated communication between protists and bacteria (Schulz-Bohm *et al.* 2017a). Interestingly, soil protists such as *Dictyostelium discoideum* (Chen *et al.* 2016) produce volatile terpenes. These terpenes might be involved in defense mechanisms, for example, to repel nematode predators. Similarly, it was shown that soil bacteria can produce specific volatiles to repel protist predators (Kai *et al.* 2009; Schulz-Bohm *et al.* 2017a).

Besides bacterivorous protists, obligate and facultative mycophageous (fungus grazing) protists are common soil inhabitants (Geisen 2016). Mycophageous protists feed mostly on yeast and fungal spores while some specialists are able to graze directly on the hyphae of filamentous fungi (Geisen *et al.* 2016). It is well known that soil fungi such as yeast produce a wide set of volatile compounds involved in various belowground interactions (Effmert *et al.* 2012; Werner *et al.* 2016). Thus, although not demonstrated yet, it is plausible that fungal volatiles might play an important role in belowground communication between soil fungi and protists, as well.

VOCs in Microbe-Plant Interactions

In recent years, evidence supporting the idea that plants respond strongly to mVOCs has grown. Most of the research carried out so far has investigated the impact of microbial VOCs on the model plant *Arabidopsis thaliana*. This has revealed that, without physical contact, microorganisms are able to drastically alter plant root system development, plant physiology, hormonal pathways, and biomass production (Ryu *et al.* 2004; Blom *et al.* 2011; Wenke *et al.* 2012; Bailly *et al.* 2014; Bitas *et al.* 2015; Ditengou *et al.* 2015; Li *et al.* 2016; Piechulla *et al.* 2017). mVOCs can also function as a direct source of nutrients for plants (Meldau *et al.* 2013), induce resistance to pathogens in plants (D'Alessandro *et al.* 2014; Kottb *et al.* 2015; Song *et al.* 2015b; Wintermans *et al.* 2016), affect plant secondary metabolite production (Santoro *et al.* 2011), directly inhibit plant pathogens (Kai *et al.* 2009; Garbeva *et al.* 2014b; De Vrieze *et al.* 2015; Kottb *et al.* 2015) and induce soil fungistasis and suppressiveness (Garbeva *et al.* 2011a; Van Agtmaal *et al.* 201). Moreover, one single mVOC can show various functions, such as dimethyl disulfide, which improves plant growth by enhancing the availability of reduced sulfur (Meldau *et al.* 2013). It also protects tobacco and corn plants against *Botrytis cinerea* and *Cochliobolus heterostrophus* by directly inhibiting pathogens and inducing systemic resistance in plants (Huang *et al.* 2012a).

Likewise, a characteristic compound of *Trichoderma asperellum*, 6-pentyl-pyrone, can increase plant defense reactions and at the same time decrease *B. cinerea* and *Alternaria alternata* sporulation (Kottb *et al.* 2015).

Many independent studies revealed that mVOCs emitted by beneficial soil microorganisms can affect plant growth but only few studies focused on how VOCs produced by soil-borne plant pathogens affect plant growth and development. These studies suggest that mVOCs from plant pathogens may modulate the trade-off between plant growth, development and defense. Bitas *et al.* (2015) showed that VOCs emitted by pathogenic *F. oxysporum* promoted the growth of *A. thaliana* and *Nicotiana tabacum* and affected auxin transport and signaling. VOCs emitted by the pathogen *Alternaria alternaria* enhanced growth, early flowering and photosynthesis rates of *Arabidopsis*, maize and pepper by affecting the levels of plastidic cytocinin (Sánchez-López *et al.* 2016). A more recent study showed that the soil-borne pathogen *Rhizoctonia solani* produced an array of mVOCs that promote plant growth, accelerate development, change plant VOCs emission and reduce insect resistance (Cordovez *et al.* 2017). This must be a successful strategy for the pathogenic fungi since with increased root biomass and stimulation of lateral root formation there is a greater surface area for fungal colonization and infection.

When analyzing mVOCs effects on plant growth, it is important to take into account, that microorganisms can produce high amounts of CO_2 that can promote plant growth (Kai & Piechulla 2009; Piechulla *et al.* 2017). Hence, a good experimental setup with appropriate controls are required to avoid artifacts in the results (Piechulla *et al.* 2017).

Alternatively, plants are able to mediate the belowground plant–microbe interactions via root-emitted VOCs (Wenke *et al.* 2010). Root-derived VOCs may serve multiple roles such as carbon sources, defense metabolites and chemo-attractants (Van Dam *et al.* 2016). Rhizobacteria such as *Pseudomonas fluorescens* and *Alcaligenes xylosoxidans* have been shown to metabolize α- pinene as their sole carbon source (Kleinheinz *et al.* 1999). Del Giudice *et al.* (2008) also reported that bacteria associated with the roots of vetiver grass (*Vetiveria zizanioides*) use sesquiterpenes as a carbon source. Undoubtedly, plants and soil microorganisms are engaged via VOCs in long-distance interactions (Van Dam *et al.* 2016). However, so far, limited knowledge exists concerning the role of plant VOCs in attracting beneficial organisms and how plant-associated microorganisms affect the quantity and quality of plant volatile emission. Only recently, using a glass olfactometer system, the attraction of distant soil bacteria by VOCs emitted by plant roots was revealed (Schulz-Bohm *et al.* 2017b). Olfactometer systems have been used successfully to study aboveground plant–herbivores interactions (Ballhorn & Kautz 2013) or belowground plant–nematode interactions (Rasmann *et al.* 2005). However, this is the first case to apply an

olfactometer to study plant–microbe interactions. Moreover, the same study revealed that upon fungal infection, the blend of root VOCs changed and specific bacteria with antifungal properties were attracted (Schulz-Bohm *et al.* 2017b).

mVOCs-Mediated Dialog

Several reports describe the chemical dialog between microbes, plants, and other organisms by the exchange of soluble compounds (Badri *et al.* 2009; Lira Jr *et al.* 2015; Song *et al.* 2015a; Liu *et al.* 2016). Most of the studies reporting mVOCs-mediated communication belowground focus on the uni-directional responses and only a few studies reported on bi-directional mVOCs-mediated interactions. For instance, the importance of mVOCs in the dialog between the fungal plant pathogen *Verticillium longisporum* and its bacterial antagonist *Paenibacillus polymyxa* was recently revealed in both *in vitro* and *in planta* experiments (Rybakova *et al.* 2017). Both microorganisms responded to one another's VOCs and this specific mVOCs-mediated interaction resulted in the inhibition of cellular metabolism and growth reduction of the fungal pathogen.

A VOCs-mediated dialog between bacteria and fungi was also reported by Spraker *et al.* 2014where VOCs of the fungal plant pathogen *Aspergillus flavus* reduced the production of the major virulence factor EPS of the bacterial plant pathogen *R. solanacearum*. In parallel, *A. flavus* responded to VOCs of *R. solanacearum* by reducing conidia production and increasing aflatoxin production.

Conclusion and Outlook

Over the last decades, our understanding of the chemical complexity of mVOCs produced by many different soil microorganisms has grown. It is clear that these small and odorous molecules can modify the behavior and promote or inhibit growth of neighboring organisms (Figure 2.1).

Most existing studies on mVOCs are focused on describing the uni-directional effect of mVOCs produced by a single organism and the responses of the organisms perceiving them without considering mVOCs-mediated dialog and the bi-directional responses to one another. Furthermore, microbial interactions taking place belowground are far more complex than single one-to-one interactions and involve more organisms, which can significantly affect mVOCs emission. For example, fungal-associated bacteria have been

shown to affect the production of VOCs in fungi (Schulz-Bohm *et al.* 2016; Splivallo *et al.* 2015) and in addition, they can affect the fungal plant-pathogenicity and repress the expression of fungal virulence genes (Minerdi *et al.* 2009). Therefore, a holistic approach considering the effect of mVOCs on belowground soil community is needed. For instance, using a metagenomics approach Yuan *et al.* (2017) revealed that mVOCs could alter the composition of soil bacterial and fungal communities and significantly increased the relative abundance of *Proteobacteria, Bacteroidetes, Firmicutes*, and *Ascomycota*. Furthermore, mVOCs influenced genes involved in important soil functions such as N-fixation (*nif*H), nitrification (*amo*A), denitrification (*nir*S) and antibiotic production (NRPS) (Yuan *et al.* 2017).

From the current scientific literature, it is clear that the most studied belowground mVOCs-mediated interactions are the interactions between bacteria, fungi and plants (Figure 2.1). There is a lack of knowledge relating to the emission of VOCs by protists, archaea or other rhizosphere organisms, such as nematodes or earthworms, indicating that these groups are currently understudied with regards to this aspect.

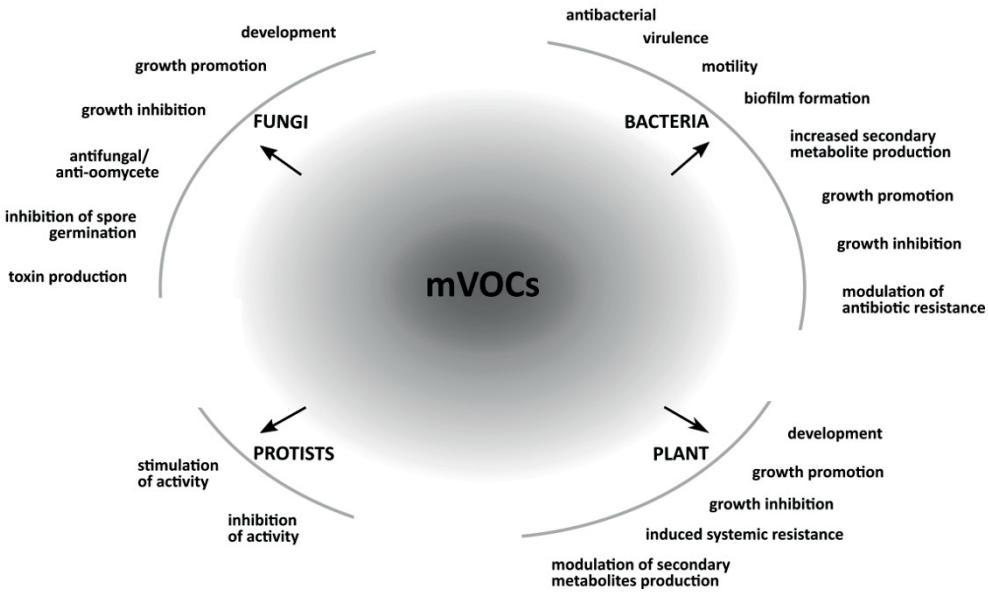

Figure 2.1. Responses in bacteria, fungi, protists and plants caused by microbial volatile organic compounds (mVOCs). The figure shows examples of responses caused my mVOCs in bacteria, fungi, protists, and plants.

Several VOCs are commonly produced and emitted by both plant roots, fungi, bacteria and protists and it is possible that these compounds act as a 'lingua franca' for intra- and inter-kingdom communication between these organisms. Let us take as an example only one chemical class, the terpenes. Terpenes are the largest and most diverse class of metabolites known to date. They are best known to humans as plants metabolites. However, recent studies revealed that terpenes can be produced by all kingdoms of life including prokaryotes (Takamatsu *et al.* 2011; Yamada *et al.* 2012; Yamada *et al.* 2015; Song *et al.* 2015b; Chen *et al.* 2016). Recently, Yamada *et al.* (2015) described a powerful bioinformatics method based on the use of Hidden Markov Models (HMMs) and Protein Families Database (PFAM) search that has allowed the discovery of terpene synthases of bacterial origin and showed that phylogenetically different bacteria can be a rich source of terpenes. Both the number, the wide distribution, and the structural diversity of terpenes provide enormous potential for mediating significant chemical interactions and communication belowground. Examples of terpene-mediated microbial interactions are presented in Figure 2.2 and Table 2.1 indicating the ecological importance of terpenes in interactions between soil micro- and macro-organisms, including plant roots.

Despite the rapid increasing numbers of studies showing the importance of mVOCs in the long-distance belowground chemical interactions, we still do not know exactly how VOCs are recognized and perceived. VOCs receptors or other perception mechanisms have not been identified in any of the described cases. The big challenge is to determine whether VOCs are internalized and transduced by receptor-mediated processes, whether they interact with the cell membrane to initiate signal transduction cascades or whether they are simply taken up by the cell and metabolized (Widhalm *et al.* 2015; Adebesin *et al.* 2017; Tissier *et al.* 2017). For plants, the current view is that due to their lipophilic nature, VOCs such as mono- and sesquiterpenes may interfere with membrane structures, thereby causing depolarization of the membranes and triggering Ca^{2+}-signaling in plants (Maffei *et al.* 2001; Heil & Land 2014). For further deciphering of mVOC-mediated microbe-microbe interactions, the mVOCs microbial perception mechanism needs to be elucidated. The application of methods for screening of mutant strains may be useful for that purpose, to identify microbial genes and proteins that are required for VOCs perception.

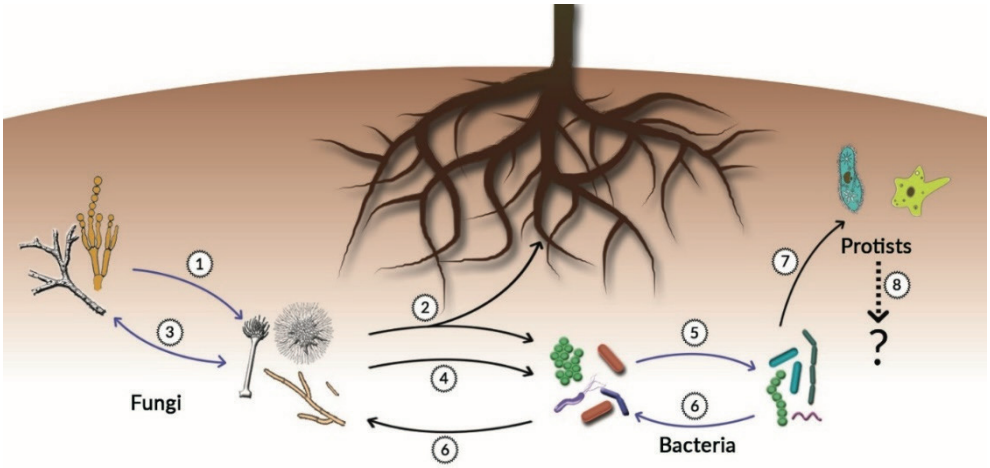

Figure 2.2. Terpenes-mediated belowground interactions. The figure shows examples of interactions between different organisms in the rhizosphere that are mediated by microbial terpenes. Blue arrows indicate intra-kingdom interactions while black arrows indicate inter-kingdom interactions. The numbers in the figure correspond with the numbers in Table 2.1.

Table 2.1. Examples of terpenes involved in belowground microbial interactions.

Origin	Nr	Compound	Biological activity	References
Fungal	1	α –Humulene	Antimicrobial (antifungal)	Minerdi *et al.* 2009
	2	β -Caryophyllene	Antimicrobial (antibacterial) Plant growth promotion	Huang *et al.* 2012b; Minerdi *et al.* 2011
	3	Farnesol	Infochemical	Hornby *et al.* 2001; Martins *et al.* 2007
	4	β-Phellandrene	Affects motility	Schmidt *et al.* 2017
Bacterial	5	Albaflavenone	Antimicrobial (antibacterial)	Gürtler *et al.* 1994
	6	β-Pinene	Antimicrobial (antifungal, antibacterial)	Garbeva *et al.* 2014b; Song *et al.* 2015b
	7	Volatile terpenes from *Collimonas*	Stimulation of protists activity	Schulz-Bohm *et al.* 2017a
Protist	8	(E,E)- α-farnesene β-barbatene	Unknown	Chen *et al.* 2016

Another big challenge is to determine what concentrations of mVOCs are produced in soil and at what distances these mVOCs are eliciting a biological response in other organisms. There is the possibility that, similar to the roles of antibiotics in nature (Davies *et al.* 2006; Yim *et al.* 2006; Romero *et al.* 2011), mVOCs could have concentration-dependent function either as weapons in intercellular chemical warfare or as signaling compounds when they are present in low concentrations.

Concerning the implementations of mVOCs, our knowledge on the potential use of those compounds in large-scale agriculture and horticulture is still limited. In agriculture systems, mVOCs have to be applied under open-field conditions, which are very different from the *in vitro* conditions currently used in most studies. There are very few studies assessing the effects of mVOCs application under open conditions and they have been summarized in a recent review from Chung *et al.* 2016. Since it was discovered that the 2,3-butanediol elicited plant growth and induced systemic resistance (Ryu *et al.* 2003; Ryu *et al.* 2004), several studies have applied this compound or the producing strains to the soil of open fields to test its effects under agricultural conditions and have revealed promising results (Velivelli *et al.* 2015). Dimethyl disulfide, frequently emitted by many bacteria, is another compound used in recent years in the novel soil fumigant PALADIN® that targets nematodes and soil-borne pathogens. However, the research concerning the application of other mVOCs in agriculture is still in its infancy. We now live in a time in which the old methods of using chemicals to protect crops need to be replaced with and, in some cases, complemented by green solutions. The traditional harmful synthetic fungicides currently used could be replaced with the so far under-explored and unique mVOCs for which significant proof of plant growth promoting effects and plant protection ability already exists. In spite of the obvious potential of mVOCs in agriculture, the field suffers from the common 'translational gap' because of a lack of studies evaluating other unexpected effects of those bioactive molecules on non-target beneficial soil organisms.

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CHAPTER 3

A Fragrant Neighborhood: Volatile Mediated Bacterial Interactions in Soil

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Abstract

There is increasing evidence that volatile organic compounds (VOCs) play essential roles in communication and competition between soil microorganisms. Here we assessed volatile-mediated interactions of a synthetic microbial community in a model system that mimics the natural conditions in the heterogeneous soil environment along the rhizosphere. Phylogenetic different soil bacterial isolates (*Burkholderia sp.*, *Dyella sp.*, *Janthinobacterium sp.*, *Pseudomonas sp.*, and *Paenibacillus sp.*) were inoculated as mixtures or monoculture in organic-poor, sandy soil containing artificial root exudates (ARE) and the volatile profile and growth were analyzed. Additionally, a two-compartment system was used to test if volatiles produced by inter-specific interactions in the rhizosphere can stimulate the activity of starving bacteria in the surrounding, nutrient-depleted soil. The obtained results revealed that both microbial interactions and shifts in microbial community composition had a strong effect on the volatile emission. Interestingly, the presence of a slow-growing, low abundant *Paenibacillus* strain significantly affected the volatile production by the other abundant members of the bacterial community as well as the growth of the interacting strains. Furthermore, volatiles released by mixtures of root-exudates consuming bacteria stimulated the activity and growth of starved bacteria. Besides growth stimulation, also an inhibition in growth was observed for starving bacteria exposed to microbial volatiles. The current work suggests that volatiles produced during microbial interactions in the rhizosphere have a significant long distance effect on microorganisms in the surrounding, nutrient-depleted soil.

Keywords:

volatiles, inter-specific interactions, rhizosphere, synthetic microbial communities, low-abundant bacteria, soil microcosms

Introduction

Microorganisms produce a great variety of secondary metabolites including antibiotics, toxins, pigments and others. Interestingly, small molecular mass metabolites such as volatile organic compounds (VOCs) were for a long time overlooked. Research of the last decades demonstrated that bacteria produce a large set of VOCs (Kai *et al.* 2009; Insam & Seewald 2010, Effmert *et al.* 2012, Bitas *et al.* 2013, Peñuelas *et al.* 2014). However, the knowledge about the biological and ecological function of those volatiles is still limited. Similar to well-studied plant VOCs, it can be assumed that VOCs released by bacteria perform diverse and crucial functions (Bitas *et al.* 2013). Recent studies revealed that bacterial volatiles can inhibit the growth of fungi or bacteria (Wrigley 2004; Kai *et al.* 2007; Vespermann *et al.* 2007; Zou *et al.* 2007; Weise *et al.* 2012; Garbeva *et al.* 2014b) and in some cases they can even function as growth-promoting agent (Wheatley 2002; Horii & Ishii 2006; Garbeva *et al.* 2014a). Additionally, volatiles emitted by bacteria can influence the metabolism of other surrounding bacteria (Kai *et al.* 2009; Garbeva *et al.* 2014a). Most studies, however, are performed *in vitro* on semi-solid media using nutrient rich conditions and may not represent the natural conditions in the microbial environment. Culture conditions including nutrient availability (Kai *et al.* 2009; Insam & Seewald 2010) and the type of incubation medium substantially affect the spectrum of released VOCs (Weise *et al.* 2012). Thus, bacteria produce a different set of volatiles when incubated in soil as compared to incubations on agar plates (Garbeva *et al.* 2014b).

Soil is a complex, nutrient-poor and highly heterogeneous environment consisting of both water- and air-filled pores (Young *et al.* 2008). Due to their physical properties such as low molecular weight (< 300 D), lipophilicity, high vapour pressure and low boiling points (Effmert *et al.* 2012; Bitas et al. 2013; Lemfack *et al.* 2014), VOCs can diffuse through water- and gas-filled pores in soil and rhizosphere environments. Therefore, VOCs can act over a wider range of scale and may play essential roles in the communication and competition between physically separated microorganisms in soil (Kai *et al.* 2009; Effmert *et al.* 2012; Garbeva *et al.* 2014a). Soil microbes occur generally in multi-species communities. However, our current knowledge on the ecological role of microbial volatiles is based mostly on studies focusing on volatiles released by (non-interacting) monocultures. During inter-specific interactions, the production of various secondary metabolites can be triggered (e.g. Garbeva & De Boer 2009; Traxler *et al.* 2013) and these secondary metabolites can act differently as compared to metabolites released by species in monoculture (Garbeva *et al.* 2014a; Tyc *et al.* 2014).

In the present study, we aim to reveal new insights into the ecological role of VOCs in microbial interactions by assessing multi-species interactions of a synthetic microbial

community in a soil model system that reflects the natural conditions in the heterogeneous soil environment along the rhizosphere. The main research questions to address were: (1) what is the role of microbial interactions and shifts in microbial community composition on volatile emission in the rhizosphere and (2) can volatiles produced during bacterial interactions in the rhizosphere stimulate the activity of starving soil microbes in the surrounding environment?

Overall, our results demonstrated that both microbial interactions and shifts in microbial community composition had a significant effect on the volatile emission and that the presence of slow growing, non-abundant bacterial species influenced the volatile production of the bacterial community. Furthermore, our results revealed that volatiles released by microbial interactions in the rhizosphere have a long distance effect on the surrounding non-active microbial community in the nutrient-depleted soil.

Material and Methods

Bacterial model strains and growth media

Bacterial strains (Table 3.1) were previously isolated from the rhizosphere of sand sedge (*Carex arenaria*) in different sandy dune soil sites (De Ridder-Duine *et al.* 2005) and characterized by 16S rRNA gene sequencing (Tyc *et al.* 2014). All bacterial isolates were pre-cultured from frozen glycerol stocks on 0.1 strength tryptone soya broth (TSB) agar (De Boer *et al.* 2007). Overnight cultures in 0.1 TSB medium were prepared prior to each microcosm experiment.

Rhizospheric soil microcosms

Sandy soil of low carbon-content (Figure S3.1) and low amount of mineral nitrogen (0.2 mg/ kg nitrite and nitrate) and phosphate (1.1 mg/ kg) was collected from an old river dune site near the village Bergharen (51°10' N, 05°40' E) in the Netherlands. The soil was dried, sieved (ø 2 mm), and gamma-sterilized by Synergy Health Ede B.V. (Netherlands). Before each microcosm experiment, the gamma-sterilized soil was acclimatized for 5 days under sterile conditions. Artificial Root Exudates (ARE) stock solution (7.5 mg carbon per ml, C/N 20.6) containing 18.4 mM glucose, 18.4 mM fructose, 9.2 mM sucrose, 9.2 mM citric acid, 4.6 mM fumaric acid, 20.5 mM lactic acid, 6.9 mM malic acid, 13.8 mM succinic acid, 2.0 mM cysteine, 6.1 mM L-alanine, 2.0 mM L-argenine, 3.6 mM L-glutamic acid, 6.1 mM L-serine, and 10 mM KH_2PO_4 was freshly prepared or stored at -20°C. The composition of the

ARE stock solution was based on previously published ARE-mixes (Griffiths *et al.* 1999; Baudoin *et al.* 2003; Garbeva *et al.* 2014b) and adapted to root-exudate composition found for the sedge *Schoenus unispiculatus* (Shane *et al.* 2006) belonging to the same family Cyperaceae as *C. arenaria.* Liquid inoculums consisting of bacteria (10^8 cells ml^{-1} per strain), ARE stock solution (pH 6.5), and phosphate buffer (10 mM KH_2PO_4, pH 6.5) were prepared. The inoculums were thoroughly mixed with the soil to establish rhizospheric soil microcosms containing 136 µg carbon per g soil and 1×10^5 CFU per g soil for each strain. The initial carbon concentration in the soil microcosms was adjusted to estimated daily carbon inputs by roots of 100-1500 µg carbon per g rhizosphere soil (Trofymow *et al.* 1987, Cheng *et al.* 1996). Control microcosms consisted of sterile soil, phosphate buffer and ARE stock solution without bacterial inoculation. The moisture content was 5.7% (w/w). Microcosms were incubated at 20°C.

Table 3.1. Bacterial strains used in this study. All strains are from organic poor, sandy dune soils in the Netherlands.

Bacterial strain (Accesion number)	Origin	Phylum (Family)
Achromobacter spp. 58-38 (KC888968)	River dune near Bergharen, Gelderland *(De Boer et al., submitted)*	Beta-Proteobacteria *(Alcaligenaceae)*
Bosea sp. AD132 (KJ685339)	Coastal outer dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	Alpha-Proteobacteria *(Bradyrhizobiaceae)*
Burkholderia sp. AD024 (KJ685239)	Dune grassland near Ouddorp, Zeeland *(De Ridder-Duine et al. 2005)*	Beta-Proteobacteria *(Burkholderiaceae)*
Dyella sp. AD056 (KJ685269)	Drift sand near Loon op Zand, Brabant *(De Ridder-Duine et al. 2005)*	Gamma-Proteobacteria (*Xanthomonadaceae*)
Janthinobacterium sp. AD080 (KJ685292)	Coastal outer dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	Beta-Proteobacteria *(Oxalobacteraceae)*
Microbacterium sp. AD141 (KJ685346)	Costal outer dunes of EastTerschelling *(De Ridder-Duine et al. 2005)*	Actinobacteria (*Microbacteriaceae*)
Paenibacillus sp. AD087 (KJ685299)	Pine plantation near Loon op Zand, Brabant *(De Ridder-Duine et al. 2005)*	Firmicutes (*Paenibacillaceae*)
Pedobacter sp. V48 (NZ_AWRU00000000)	Coastel dune site Terschelling *(De Boer et al. 1998)*	Bacteroidetes *(Sphingobacteria)*
Pseudomonas sp. AD021 (DQ778036)	Coastel inner dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	Gamma-Proteobacteria *(Pseudomonadaceae)*
Rhizobium sp. 45-29 (KC888976)	River dune near Bergharen, Gelderland *(De Boer et al., submitted)*	Alpha-Proteobacteria *(Rhizobiaceae)*

Bioassay for the selection of bacterial strains

Three soil microcosms for each bacterial strain were set up in 25 ml gas tight glass bottles as described above. The head space in the bottles was filled with atmospheric gas. All microcosms were incubated for 7 days at 20°C and the bottles were flushed with fresh air every two days. Gas samples were taken before and after flushing with fresh air and the CO_2 production was measured with an Ultra GC gas chromatograph (Interscience, The Netherlands) equipped with a flame ionization detector (FID) and a Rt-QBond (30 m, 0.32 mm, ID; Restek, USA) capillary column. Carrier gas was helium at a flow rate of 5 ml min^{-1}. Injector, oven and detector temperatures were 150°C, 50°C, and 350°C, respectively. CO_2 concentrations in the headspace were calculated based on external gas standards (Westfalen AG, Germany) and the ideal gas law.

Soil samples were regularly taken to monitor the bacterial growth. For this 1 g of soil was mixed with 9 ml phosphate buffer (pH 6.5) in 20 ml Greiner tubes. The tubes were shaken on a rotary shaker at 180 rpm for 30 min at 20°C and serial dilutions were plated on 0.1 TSB agar. All plates were incubated at 20°C and colony forming units (CFU) were counted after 3 days.

Collection of bacterial volatiles and monitoring of bacterial growth in rhizospheric soil microcosms

For the collection of VOCs, rhizospheric soil microcosms were established as indicated above in glass Petri-dishes with an exit at the top to which a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) could be fixed. In the rhizospheric soil microcosms, the bacterial strains *Burkholderia* sp. AD024, *Dyella* sp. AD056, *Janthinobacterium* sp. AD080, *Pseudomonas* sp. AD021, and *Paenibacillus* sp. AD087 were either incubated as monocultures or as mixtures of four (without *Paenibacillus sp*. AD087) or five strains. The growth of the strains was monitored by quantification of bacterial 16S rRNA genes from extracted DNA of soil samples (three per microcosm) taken at the beginning of the experiment and after 96 h incubation. Soil samples were stored at -80°C until nucleic acid extraction (see below). VOCs produced by the monocultures, bacterial mixtures and control (see above) were trapped for 24 h after 3 days of incubation. Traps were removed, capped and stored at 4°C until analysis.

VOCs analysis

VOCs were desorbed from the traps using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., UK) at 210°C for 12 min (He flow 50 ml/min).

The desorbed VOCs were subsequently collected on a cold trap at -10°C and introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, USA) by heating the cold trap for 3 min to 280°C. Split ratio was set to 1:201, and the column used was a 30 × 0.25 mm ID RXI-5MS with as film thickness of 0.25 µm (Restek 13424-6850, Bellefonte, PA, USA). The temperature program was as follows: 2 min at 39°C, 3,5°C/min to 95°C, 6°C/min to 165°C, 15°C/min to 250°C, and finally 40°C/min to 300°C which was hold for 20 min. VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (30–400 AMU, 4 spectras/s). GC/MS data were collected and converted to mzData file using the Chemstation B.06.00 (Agilent Technologies, USA). Data were further processed with MZmine 2.14.2 (Pluskal *et al.* 2010) with the tools mass detection (centroid mode, noise level = 1000), chromatogram builder (Min time span = 0.04 min, Min height = 1.8E04-2.5E04, m/z tolerance of 1 m/z or 5 ppm), and chromatogram deconvolution (local minimum search, chromatographic threshold = 40 %, Min in RT range = 0.1 min, Min relative height = 2.0 %, Min absolute height = 1.5E04, Min ratio of peak top/edge = 2, peak duration = 0.0-0.7 min). Detected and deconvoluted peaks were identified by their mass spectra using NIST MS Search and NIST 2014 (National Institute of Standards and Technology, USA, http://www.nist.gov) and aligned by RANSAC aligner (mz tolerance = 1 m/z or 5 ppm, RT tolerance = 0.1, RT tolerance after correction = 0.05, RANSAC iteration = 10000, Min number of points = 60 %, threshold value = 0.1). Processed data were exported for further statistical analysis (see below). The identification of detected compounds was further evaluated by using the software AMDIS 2.72. The retention indexes were calculated for each compound and compared with those found in NIST 2014 and in house databases. Detected compounds were named as identified compounds if the Match and Reverse-Match factor of the listed compound in the NIST and in house NIOO-library was higher than 800, the spectra of the detected compound matches the one of the listed compound, and the difference between the retention index calculated for the detected compound and of the listed compound (for a semi-standard non-polar column) was not bigger than four. The Match and Reverse match factor refer to the similarity of the mass spectrum of the sample with the library spectrum of the Hit (Watson & Sparkman 2013). Some identified compounds were also verified by co-injection of pure compounds (Table 3.2).

Table 3.2. Volatile organic compounds produced by a bacterial mixture of five strains (*Burkholderia sp.* AD024, *Dyella sp.* AD056, *Janthinobacterium sp*. AD080, *Pseudomonas sp*. AD021, and *Paenibacillus sp*. AD087), referred to as 5-Mix, or four strains excluding *Paenibacillus sp.* AD087, referred to as 4-Mix. Bacteria were incubated for four days in soil supplied by ARE.

Compound	Class	RI
Compounds detected for 4-Mix and 5-Mix		
2-Pentanone[a,c]	Ketone	683
3-Pentanone[a,c]	Ketone	695
Unknown[c]	n.s.	709
*Dimethyl disulfide[b,c,e]	Organosulfur	744
2-Octanol[a,c]	Alcohol	996
L-Fenchone[a,c]	Monoterpene	1088
Unknown[d]	n.s.	1091
Camphor	Terpenoid	1144
Unknown	n.s.	1316
2,6-Bis(1,1-dimethylethyl)-2,5-Cyclohexadiene-1,4-dione	Aromate/ Phenol	1461
Butylated Hydroxytoluene[a,b,c,d,e]	Aromate	1501
Unknown[c]	n.s.	1515
2,2,4-Trimethyl-1,3-pentanediol diisobutyrate[a,e]	Ester	1588
Unknown[b,c,d]	n.s.	1718
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester[a,b]	Ester	1874
Compounds detected for 4-Mix		
Methoxy-acetaldehyde[b,d]	Aldehyde	559
Cyclopentene[a,b]	Alkene	570
tert-Butanol[a,b,c,e]	Alcohol	575
Acetic acid	Organic acid	593
2-methyl-2-propen-1-ol[a,b,c]	Alcohol	605
Ethyl benzene	Aromate	857
3-Heptanol[b,c,e]	Alcohol	896
n-Hexadecanoic acid	Organic acid	1964
Compounds detected for 5-Mix		
Sulfur dioxide[a]	Organosulfur	548
1,3,5-Trifluorobenzene[a,b]	Aromate	624
Propanoic acid, 2,2-dimethyl-, methyl ester[b]	Ester	720
2-Hexanone[c,d]	Ketone	790
2-Heptanone[c]	Ketone	888
Unknown	n.s.	1020
Methyl 2-ethylhexanoate	Ester	1039

Table 3.2 continued.

Compound	Class	RI
Compounds detected for 5-Mix		
Endo-borneol[a,b,d]	Terpene	1167
*2,5-Bis(1-methylethy)-pyrazine	Aromate	1185
Unknown	n.s.	1200
Unknown	n.s.	1231
Unknown[a,c]	n.s.	1244
Unknown	n.s.	1283
Unknown	n.s.	1343
Unknown[a,c]	n.s.	1690
*Docosane[b]	Alkene	2200

a Volatiles also detected for monocultures of *Burkholderia sp.* AD024;
b Volatiles also detected for monocultures of *Dyella sp.* AD056.
c Volatiles also detected for monocultures of *Janthinobacterium sp.* AD080.
d Volatiles also detected for monocultures of *Paenibacillus sp.* AD087.
e Volatiles also detected for monocultures of *Pseudomonas sp.* AD021.
RI Linear retention Index of a 30 × 0.25 mm ID RXI-5MS column.
n.s. not specified.
* Verified by co-injection of pure compound.

Bioassay to test the effect of microbial VOCs on nutrient-limited bacteria in soil

Soil microcosms containing the bacterial strains *Burkholderia* sp. AD024, *Dyella* sp. AD056, *Janthinobacterium* sp. AD080, *Pseudomonas* sp. AD021, and *Paenibacillus* sp. AD087 were set up in two-compartment Petri-dishes (Figure 3.3 A and S3.3 A). Per Petri-dish, one nutrient-limited compartment was established containing 20 g gamma-sterilized soil mixed with 1 x 10^5 CFU per g soil of each strain and phosphate buffer (10 mM KH_2PO_4, pH 6.5). The second compartment was either filled with a mix of 20 g soil, phosphate buffer and ARE stock solution (138 µg carbon per g soil), referred to as control, or a mix of 20 g soil, the five bacterial strains (1 x 10^5 CFU per g soil of each strain), phosphate buffer and ARE stock solution, referred to as Treatment 1 or Treatment 2 (Figure 3.3 A and Figure S3.3 A). In case of Treatment 2, bacteria in the nutrient-limited compartment were pre-incubated for 2 days before the bacteria-ARE-soil-mix was added to the second compartment. It can be assumed that during the pre-incubation available carbon sources in the soil should be fully consumed so that bacteria were strongly nutrient limited before they were exposed to microbial volatiles of the second compartment. The composition of the ARE containing compartment is equivalent to the rhizospheric microcosms described above. The moisture content of all compartments was 5.8 % (w/w). All samples were set up in triplicates and incubated at 20°C for 6 days. To monitor bacterial activity and potential growth, about 1.5 g soil were taken two times per compartment at the start of the experiment and after 2, 4 and 6 days of

incubation. For nucleic acid extraction, 0.5 g of the collected soil was immediately treated with a salmon sperm DNA solution and freeze-dried at -80°C. Leftover soil collected was stored as backup at -80°C.

Nucleic acid extraction

A solution (~pH 8) of 10 mg per ml low molecular weight salmon sperm DNA (Sigma-Aldrich, The Netherlands) was prepared with DNase- and RNase-free water (Qiagen, The Netherlands). According to Paulin *et al.* (2013), 0.5 g of soil per sample was mixed with 0.5 ml of salmon sperm DNA solution in Lysing Matrix E tubes (MP Biomedicals, The Netherlands) and freeze-dried overnight at -80°C. DNA and RNA were co-extracted using the modified protocol described by Griffiths *et al.* (2000). Briefly, 500 µl of cetyl-trimethyl ammonium bromide (CTAB) buffer (10% CTAB in 0.7 M NaCl mixed with 1 volume 240 mM phosphate buffer pH 8.0), 50 µl of 2% N-lauroyl sarcosine, 50 µl of 2% sodium dodecyl sulfate, and 400 µl of phenol-chloroform-isoamyl alcohol (25:24:1, Sigma-Aldrich, The Netherlands) were added to the freeze-dried soil. A subsequent bead beating lysis for 30 s at 5.5 ms^{-1} was performed three-times followed by 7 min centrifugation at 14,000 rpm and 4°C. The resulting aqueous supernatant was mixed with 1 volume (vol.) of chloroform-isoamyl alcohol (24:1, Sigma-Aldrich, The Netherlands) and centrifuged for 5 min at 14,000 rpm and 4°C. After a second phenol extraction with 1 vol. chloroform-isoamyl alcohol the aqueous supernatant was thoroughly mixed with 2 vol. of 20 % polyethylene glycol 6000 (AppliChem, Germany) in 1.6 M NaCl, 0.1 vol of 100 mM $MgCl_2$, and 1.5 µl glycogen (20 mg ml^{-1}; Thermo Fisher Scientific, The Netherlands). Nucleic acids were precipitated for 2 h at 4°C, followed by 40 min centrifugation at 14,000 rpm and 4°C. The pellet was washed with 70% ethanol, air-dried and eluted in 37-65 µl DNase- and RNase-free water. Nucleic acid extracts were stored at -80°C or subsequently used for quantification of bacterial 16S rRNA genes (see below).

DNase treatment and reverse transcription

Nucleic acid extracts were treated according to the manufacturer's protocol with 1-2 U DNase I (Thermo Fisher Scientific, The Netherlands) and incubated for 45 min at 37°C. RNA was purified by an overnight precipitation at -20°C with 0.1 vol. ammonium acetate (2.5 mM, Sigma-Aldrich), 2.5 vol. 100 % ethanol, and 1.3 µl glycogen, followed by centrifugation for 40 min at 13,000 rpm and 4°C. The RNA pellet was washed twice with 70 % ethanol and eluted in 27 µl DNase- and RNase-free water. RNA was stored at -80°C or subsequently used for reverse transcription with SuperScript® III First-Strand Synthesis

System (Invitrogen, The Netherlands). The reverse transcription was performed according to the manufacturer's instruction. Resulting cDNA was precipitated overnight with 0.1 vol. 2 M NaCl, 2.5 vol. 100 % ethanol, and 0.7 µl glycogen at -20°C, followed by 40 min centrifugation at 13,000 rpm and 4°C. After washing with 70 % ethanol, the cDNA pellet was eluted in 25 µl DNase- and RNase-free water. cDNA was stored at -20°C or immediately used for the quantification of bacterial 16S rRNA.

Quantitative PCR (qPCR) of bacterial 16S rRNA genes and transcripts

All qPCRs were performed with a Rotor-Gene Q cycler (Qiagen, The Netherlands) whereas each template DNA or cDNA was quantified in triplicates. The 20 µl reaction mixture consisted of 1-fold SensiFAST™ SYBR® No-ROX Kit (Bioline GmbH, The Netherlands), BSA (0.5 µg μl^{-1}), 375-500 nM forward and reverse primers (Table S3.1), and 5 µl of diluted template DNA or cDNA of 2-6 ng μl^{-1}. Negative controls consisting of DNase- and RNase-free water instead of template DNA were included in every qPCR run. Conditions for the primer sets targeting 16S rRNA gene of *Burkholderia*, *Dyella*, and *Paenibacillus* (Table S3.1) were as follows: 5 min initial denaturation at 95°C ,37 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 64°C, elongation for 30 s at 72°C, and fluorescence signal detection for 15 s at 82°C. The 37th PCR cycle was followed by a melting curve analysis from 64 to 95°C with increments of 1°C. QPCR conditions for the primer sets targeting 16S rRNA gene of *Janthinobacterium* and *Pseudomonas* were the following: 5 min at 95°C, ensued by 37 cycles of denaturation for 30 s at 95°C, annealing for 20 s at 62°C, elongation for 20 s at 72°C, fluorescence signal detection for 15 s at 82°C, and a melting curve analysis from 62°C to 95°C immediately after the last qPCR cycle. Agarose gel electrophoresis of qPCR products displayed single bands of expected size (Table S3.1). Gene copy numbers were calculated according to a standard curve which was set up by serially diluting M13uni/rev PCR products of a pGEM-T vector containing a 16S rRNA gene fragment of the respected target organism (Zaprasis *et al.* 2010).

Statistical analysis

All experiments were performed in triplicates. The statistical analysis of processed GC-MS data consistent of detected mass features per sample was conducted with MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/MetaboAnalyst, Xia *et al.* 2015). Prior one-way ANOVA and multivariate analysis (PCA), data were filtered (interquantile range) and normalized (log transformation and auto scaling).

The statistical analysis on qPCR data as well as of CFU counts of *Pseudomonas sp.* AD021 growing as monoculture in soil microcosms supplied with or without ARE was performed with R 3.1.1 (http://www.r-project.org/) using ANOVA followed by Tukey's HSD test (De Mendiburu 2014). To obtain normality of errors, data were log-transformed. Significance of enhanced CO_2 production by *Pseudomonas sp.* AD021 growing in soil supplied with ARE was assessed by student's t-test. Differences revealed by statistical tests were considered significant for $P < 0.05$.

Results

Bacterial growth and volatile production in rhizospheric soil microcosms containing single or multiple species

For the rhizospheric soil microcosms, a sandy soil was selected with a low *in situ* availability of carbon. Metabolic activity and growth of bacteria were restricted without the addition of ARE to this soil (Figure S3.1). In the presence of the supplied carbon source, the growth and CO_2 production of different rhizobacterial strains was assessed (Figure S3.2). Finally, five phylogenetic different bacterial strains (*Burkholderia sp*. AD024, *Dyella sp*. AD056, *Janthinobacterium sp*. AD080, *Pseudomonas sp*. AD021, and *Paenibacillus sp*. AD087) were selected. Those strains, except for *Paenibacillus sp*. AD087, showed a similar CO_2 production profile. A maximal CO_2 production was reached after 6 days of incubation (Figure S3.2) which suggested that the supplied ARE as sole carbon source were fully consumed. Furthermore, the selected Gram-negative strains multiplied to a maximum of about 10^8 CFU per g soil in the microcosms supplied with ARE (Figure S3.2). A quantification of 16S rRNA gene copy numbers revealed that the growth of *Burkholderia sp*. AD024, *Dyella sp*. AD056, and *Pseudomonas sp*. AD021 was significantly reduced in incubations with other bacteria in the ARE supplied soil (Figure 3.1). The Gram-positive strain *Paenibacillus sp*. AD087 grew poorly in the rhizospheric soil microcosms, both in monoculture and mixture (Figure 3.1 and S3.2). However, in presence of *Paenibacillus sp*. AD087 the growth of the other bacteria in the mixture was affected. For instance, the 16S rRNA gene copy number (no.) of *Dyella sp.* AD056 and *Pseudomonas sp.* AD021 was significantly higher and for *Janthinobacterium sp.* AD080 significantly lower as compared to incubations without *Paenibacillus sp*. AD087 (Figure 3.1).

A different blend of VOCs was produced by the bacterial mixture in comparison to the monocultures (Figure 3.2). The VOCs profile was different when the bacterial mixture consisted of five or four strains without *Paenibacillus sp*. AD087 (Figure 3.2). Volatiles released by the bacterial mixture consisted of alcohols, ketones, and esters as well as

aromatic and organosulfur compounds. Some of those were also produced by the monocultures (Table 3.2). Most VOCs only released by the mixture of four bacterial strains excluding *Paenibacillus sp*. AD087 were alcohols and organic acids whereas VOCs released by for the mixture of all five strains consisted mainly of ketones, ester, and aromatic compounds. However, also numerous unknown volatile compounds were detected, especially for the bacterial mixture consisting of five strains (Table 3.2).

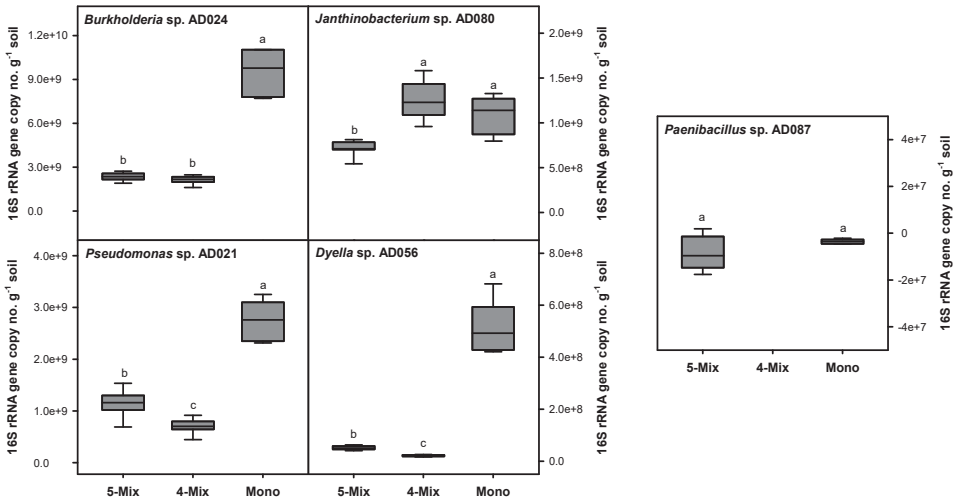

Figure 3.1. Changes in bacterial 16S rRNA gene copy numbers per g soil after 4 days of incubation. Bacteria were incubated in soil mixed with artificial root exudates as monoculture (Mono), bacterial mixture of five strains (5-Mix), or mixture of four strains excluding *Paenibacillus sp.* AD087 (4-Mix). Data represent values corrected for the starting time point t0. Different letters indicate significant difference ($P < 0.05$) between values resulted from One-way ANOVA and Tukey's HSD test.

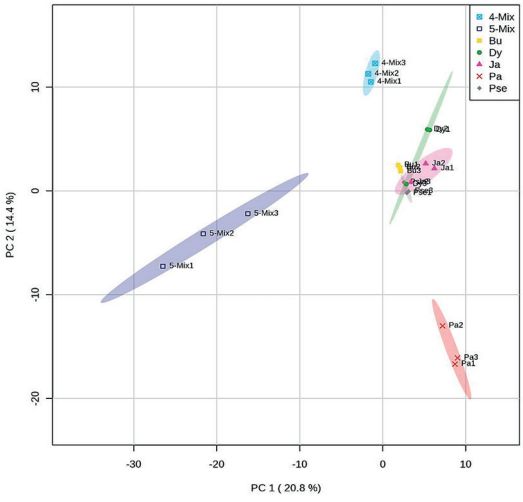

Figure 3.2. PCA score plot of volatiles produced by bacteria in soil after 4 days of incubation in soil with artificial root exudates (ARE). The bacterial strains Burkholderia sp. AD024 (Bu), Dyella sp. AD056 (Dy), Janthino-bacterium sp. AD080 (Ja), Pseudomonas sp. AD021 (Pse), and Paenibacillus sp. AD087 (Pa) were incubated as monoculture, mixture of five strains (5-Mix), or mixture of four strains excluding Paenibacillus sp. AD087 (4-Mix). Data represent multivariate analysis of mass features of volatiles only detected for microcosms containing bacteria.

Effect of microbial volatiles on starving bacteria

To test if volatiles produced during microbial interactions in a rhizosphere environment can stimulate starving microbes in the nutrient-depleted surrounding soil, soil microcosm experiments in two-compartment Petri-dishes with a mixture of all five bacterial strains were performed. Bacteria without additional carbon-source (compartment C4 of Treatment 1 and compartment C6 of Treatment 2) were exposed to volatiles produced by bacteria supplied with ARE (compartment C3 and C5, respectively) (Figure 3.3 A and Figure S3.3 A).

The 16S rRNA copy no. per g soil increased over time for bacteria supplied with ARE as well as for bacteria in the nutrient-depleted soil that were exposed to microbial VOCs (Figure 3.3 B and Figure S3.3 B). For the control microcosms, in which the ARE supplied compartment did not contain bacteria (Figure 3.3 A and Figure S3.3 A), the bacterial 16S rRNA copy no. per g soil did not significantly change over time. After 6 days of incubation, the 16S rRNA copy no. per g soil was significantly higher for all five bacterial strains in the nutrient-depleted soil exposed to microbial VOCs as compared to the control without exposure to microbial VOCs (Figure 3.3 B). In case of *Dyella sp.* AD056 and *Paenibacillus sp.* AD087, the increase in 16S rRNA copy no. per g soil after 6 days of incubation was 1.5 and 12 times higher, respectively, when starving bacteria were exposed to microbial VOCs (compartment C4) as compared to bacteria growing in the ARE containing compartment (compartment C3) (Figure 3.3 B). A similar trend was observed when bacteria were pre-incubated for 2 days in the soil before exposure to microbial VOCs (Figure S3.3 B). Hence, volatiles released by mixtures of ARE-consuming bacteria significantly affected the activity of starving bacteria without ARE, based on an increase in 16S rRNA.

The 16S rRNA gene copy no. per g soil was measured for *Burkholderia sp.* AD024, *Dyella sp.* AD056, and *Paenibacillus sp.* AD087 to determine potential growth stimulation by exposure to bacterial VOCs under starving conditions. In case of *Burkholderia sp.* AD024 and *Dyella sp.* AD056, a significantly higher increase in 16S rRNA gene copy no. per g soil was observed for starving bacteria exposed to bacterial VOCs in comparison to the control (Figure 3.3 C). In contrast, the 16S rRNA gene copy no. per g soil decreased over time for the strain *Paenibacillus sp.* AD087, even when nutrients were available or bacteria were exposed to VOCs (Figure 3.3 C). Thus, the increase in activity by exposure to bacterial VOCs is coinciding with growth for the strains *Burkholderia sp.* AD024 and *Dyella sp.* AD056 but not for the Gram-positive *Paenibacillus sp.* AD087.

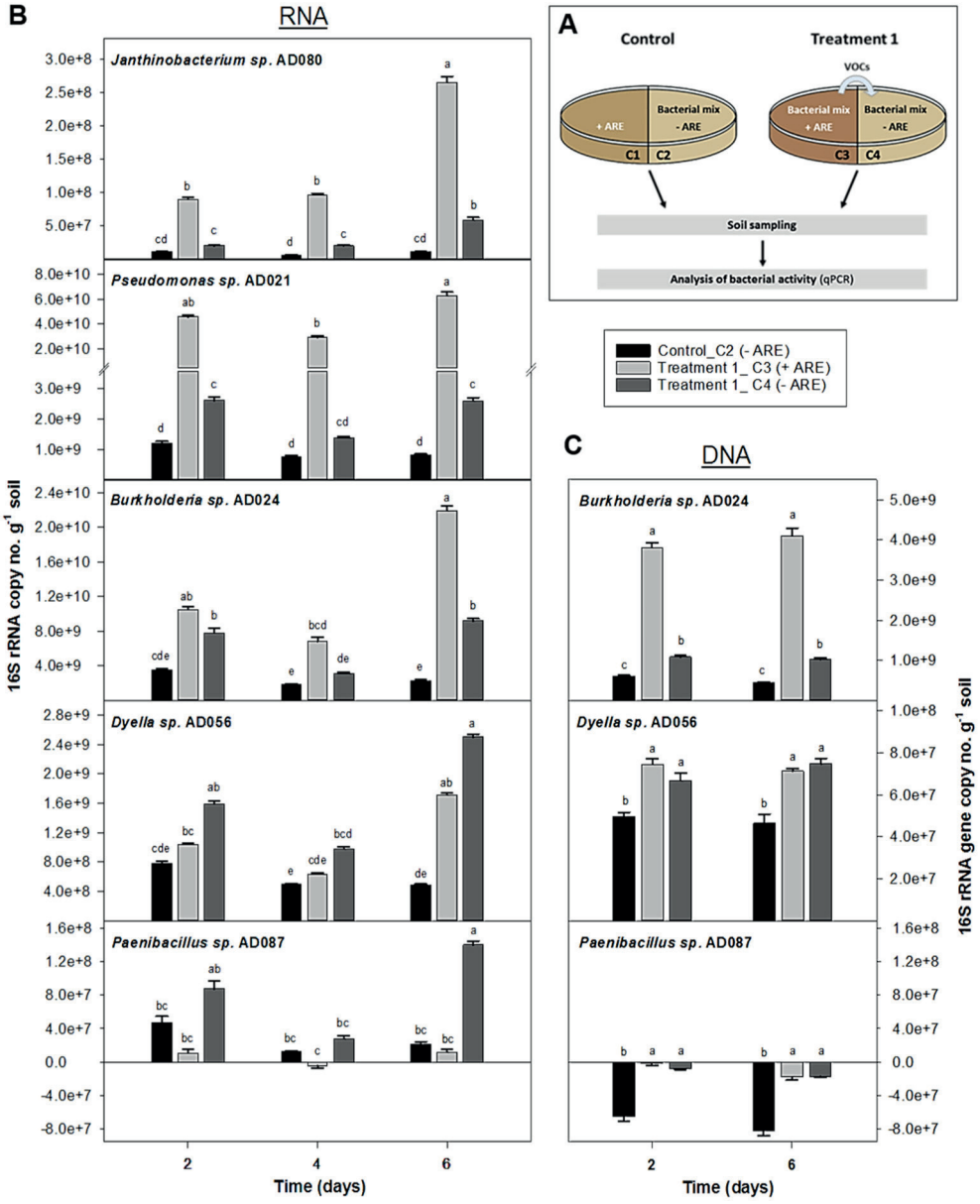

Figure 3.3. Influence of bacterial volatiles on bacteria in nutrient-depleted soil. Increase in 16S rRNA copy number (B) and 16S rRNA gene copy number per g soil (C) were determined for a bacterial community consisting of *Burkholderia sp.* AD024, *Dyella sp.* AD056, *Janthinobacterium sp.* AD080, *Pseudomonas sp.* AD021, and *Paenibacillus sp.* AD087 which was incubated in nutrient-poor soil. (A) In Treatment 1, bacteria in nutrient-depleted soil in compartment C4 (dark grey) were exposed to volatiles produced by bacteria supplied with artificial root exudates (ARE) in compartment C3 (light grey). The control compartment C2 (black) represents bacteria in nutrient-depleted soil not exposed to bacterial volatiles. Data represent mean (n = 6) and standard error corrected for the starting time point t0. Different letters indicate significant differences ($P < 0.05$) between values resulted from One-way ANOVA and Tukey's HSD test.

Discussion

Most of the current knowledge about the possible functioning of microbial volatiles is based on *in vitro* studies under nutrient rich conditions (e.g. Kai *et al.* 2007; Vespermann *et al.* 2007; Zou *et al.* 2007). This is different from the nutrient-limiting conditions with which microbes have to deal in most soil environments. Here, we developed a soil model system mimicking more closely the natural situation occurring in and around the rhizosphere to reveal new insights into the ecological role of volatiles in microbial interactions in soil.

For bacteria incubated in rhizospheric soil microcosms, it was observed that the volatiles produced by the mixture differed from those produced by each strain. Some volatiles were only emitted by the bacterial mixture and not by the monocultures. The shift in the volatile blend can be due to competitive interactions (Garbeva *et al.* 2014a). This was indicated by a significant decrease in 16S rRNA gene copy number of *Burkholderia sp.*, *Dyella sp.*, and *Pseudomonas sp.* when they were growing in mixture with other bacterial strains. Besides inter-specific competitive interactions it was also observed that shifts in the bacterial community composition, i.e. bacterial mixture consistent of four or five different strains, influenced the volatile production. Therefore, as proposed by Mc Neal and Herbert (2009) changes in the VOCs profile can be a potential indicator of microbial community composition shifts. Indeed, recent studies pointed at a relationship between the composition of soil bacterial communities and that of VOCs (Van Agtmaal *et al.* 2015; Hol *et al.* 2015). Interestingly, the volatile emission by the bacterial mixture was strongly affected by the presence of the poorly-growing, non-abundant *Paenibacillus sp.* This is in line with recent studies revealing that low abundant microbes can play an important role in ecosystem functioning (Hol et al. 2010; Lynch & Neufeld 2015; Hol *et al.* 2015). Furthermore, we observed that in the presence of the slow-growing *Paenibacillus sp.*, the 16S rRNA gene copy number of *Dyella sp.* and *Pseudomonas sp.* was significantly higher. Hence, non-abundant species can promote the growth of dominant species. On the other hand, they may also trigger the production of various growth-suppressing secondary metabolites such as antimicrobial volatiles (Jousset *et al.* 2014; Hol *et al.* 2015). In the presence of *Paenibacillus sp.*, several additional volatiles were released by the bacterial mixture which might be involved in the growth suppression of *Janthinobacterium sp.* besides non-volatile antimicrobial compounds released by the interacting bacteria. Among those volatiles 2,5-bis(1-methylethy)-pyrazine was produced. It was recently reported that *Paenibacillus* can produce pyrazines with broad spectrum antimicrobial activity against bacteria, fungi and yeast (Rybakova *et al.* 2015). Here, the pyrazine-derivate was only detected for the bacterial mixture but not for *Paenibacillus sp.* in monoculture. However, it is possible that due to

competitive interactions in the mixture the Gram-positive strain became active and started to produce the pyrazine compound.

Microorganisms are thought to use VOCs to influence other organisms living at a distance in the same soil environment (Stahl & Parkin 1996). In the present study, we tested if volatiles produced by bacterial mixtures growing on root exudates can affect the activity of starving bacteria unable to access those root exudates. While microbes in the rhizosphere benefit from a constant flow of organic substrates (Effmert *et al.* 2012) volatile compounds released from the rhizosphere can represent an important carbon source to microorganisms in the nutrient-poor surrounding soil (Owen *et al* 2007; Gramss & Bergmann 2008; Owen *et al.* 2007; Ramirez *et al.* 2009). Besides, it has been suggested that volatiles produced by rhizosphere microorganisms could act as chemo-attractants to the nutrient-rich environment around the roots (Garbeva *et al.* 2014a). Our results revealed that after 2 days of incubation, the activity of the starving bacteria *Burkholderia sp.*, *Dyella sp.*, and *Pseudomonas sp.* was already stimulated by exposure to microbial VOCs. After 6 days of incubation, the activity of all five bacterial strains in the nutrient-depleted soil was significant increased by the exposure to microbial VOCs. Thus, volatiles released by rhizosphere-inhabiting bacterial communities can stimulate the activity of the surrounding starving bacteria in nutrient-depleted soil. The mechanism behind the activation of bacteria by volatiles, however, remains unclear. It was reported that volatiles can function as growth-promoting agents (Wheatley 2002; Horii & Ishii 2006; Garbeva *et al.* 2014a) which might explain the increase in bacterial activity by exposure to microbial VOCs. Quantification of 16S rRNA gene copy number revealed that the growth of the starving *Burkholderia sp.* and *Dyella sp.* was induced by exposure to microbial VOCs. Hence, the growth of starving bacteria in soil can be promoted by volatiles released from the rhizosphere. Bacteria are able to detoxify VOCs and/or use them as carbon and energy source. For instance, it was shown that *Pseudomonas fluorescens* is able to degrade the volatile compound alpha-pinene and to use it as a sole carbon source (Best *et al.* 1987; Kleinheinz *et al.* 1999). Furthermore, *Burkholderia* was reported as a toluene-degrader (Chen *et al.* 2015) and *Dyella* was abundant on a biofilter exposed to terpenes (Moe *et al.* 2013). In the current study, various terpenes and butylated hydroxytoluene were released by the bacterial mixture supplied with artificial root exudates in soil. It can be assumed that some of these volatiles stimulated the growth of *Burkholderia sp.* and *Dyella sp.* in the nutrient-depleted soil. This, however, needs to be verified in future studies.

Interestingly, while the activity of *Paenibacillus sp*. was strongly induced by exposure to microbial VOCs the growth was suppressed. As previously reported some VOCs can act as growth-inhibiting, toxic agents (Peñuelas *et al.* 2014) and induce stress response (Kim *et*

al. 2013; Garbeva *et al.* 2014a). This might explain the strong induction in the activity and at the same time growth-suppression of *Paenibacillus sp*. by exposure to microbial VOCs. Another possible scenario for the strong increase in activity of *Paenibacillus sp*. is the induction of motility to escape from the toxic environment. A stimulation of motility by exposure to microbial VOCs was already reported for several bacterial species (Kim *et al.* 2013; Garbeva *et al.* 2014a; Hagai *et al.* 2014). For microbes living in the heterogeneous soil environment, an activation of motility by volatiles may be important to move towards nutrient-rich regions or to escape from hostile areas. Hence, volatiles in soil can provide important information on the quality of the nearby surroundings.

Besides the role as growth-promoting or growth-suppressing agents (Peñuelas *et al.* 2014), volatiles can also induce the production of non-volatile secondary metabolites. For instance, it was recently reported that microbial volatiles induce the production of inhibiting secondary metabolites in *Pseudomonas fluorescence* against Gram-positive bacteria (Garbeva *et al.* 2014a). Thus, the growth suppression of *Paenibacillus sp*. may be also due to non-volatile secondary metabolites which production was activated by microbial volatiles.

In conclusion, this study revealed that the blend of released volatiles from the rhizosphere is affected by inter-specific competitive interactions and shifts in the microbial community composition. Moreover, the presence of non-abundant, slow-growing species can strongly influence the volatile production by other dominant species. Based on our results and other recent studies it is evident that microbial volatiles in soil can serve multiple roles as C-source, defense metabolites, chemoattractant, repellants or other unknown so far. Hence, volatiles released by rhizosphere-inhabiting microbial communities can have a significant long distance effect on starving microorganisms in the surrounding, nutrient-depleted soil.

Acknowledgments

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Supplementary Material

Table S3.1. Primers and qPCR conditions used in this study.

Primers[1]	Sequence (5′ · 3′)	Size (bp)	Primer conc.[2] (nM)	Target	Reference
BKH812F	CCCTAAACGATGTCAACTAGTTG	402	500	*Burkholderia*	Bergmark *et al.* 2012
BKH1249R	ACCCTCTGTTCCGACCAT		500	*Burkholderia*	Bergmark *et al.* 2012
DY456F	CTAATATCCGGTGGGGCTGAC	267	400	*Dyella*	This study
DY722R	TTCGCCACTGATGTTCCTCC		400	*Dyella*	This study
PA1249F	GTACAACGGGCTGCGAAATC	198	400	*Paenibacillus*	This study
PA1446R	CCACCGACTTCGGGTGTTAT		400	*Paenibacillus*	This study
Pse853F	CGCATTAAGTTGACCGCCTG	174	375	*Pseudomonas*	This study
Pse1026R	ATGTTCCCGAAGGCACCAAT		375	*Pseudomonas*	This study
JA1223F	ATGGTACATACAGAGCGCCG	194	400	*Janthino-bacterium*	This study
JA1416R	CTGGTAAAACCCGCTCCCAT		400	*Janthino-bacterium*	This study

1 Forward primer is indicated by F and reverse primer by R.
2 Final primer concentrations in 20 µl qPCR reaction mix.

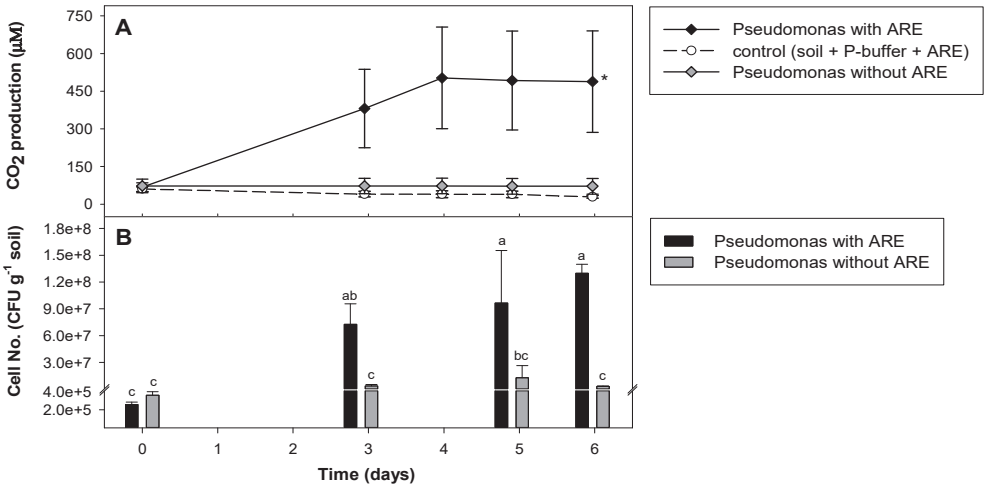

Figure S3.1. Evidence for nutrient-limited growth in soil microcosms. Cumulative CO2-production (A) and growth (B) was determined for the rhizospheric strain Pseudomonas sp. AD021. Data represent mean (n = 3) and standard error. Star indicates a significant higher CO2 production ($P<0.05$) for soil microcosms of Pseudomonas supplied with artificial root exudates (ARE). Different letters indicate significant difference ($P < 0.05$) between cell numbers (no.) resulted from one-way Anova and Tukey's HSD test.

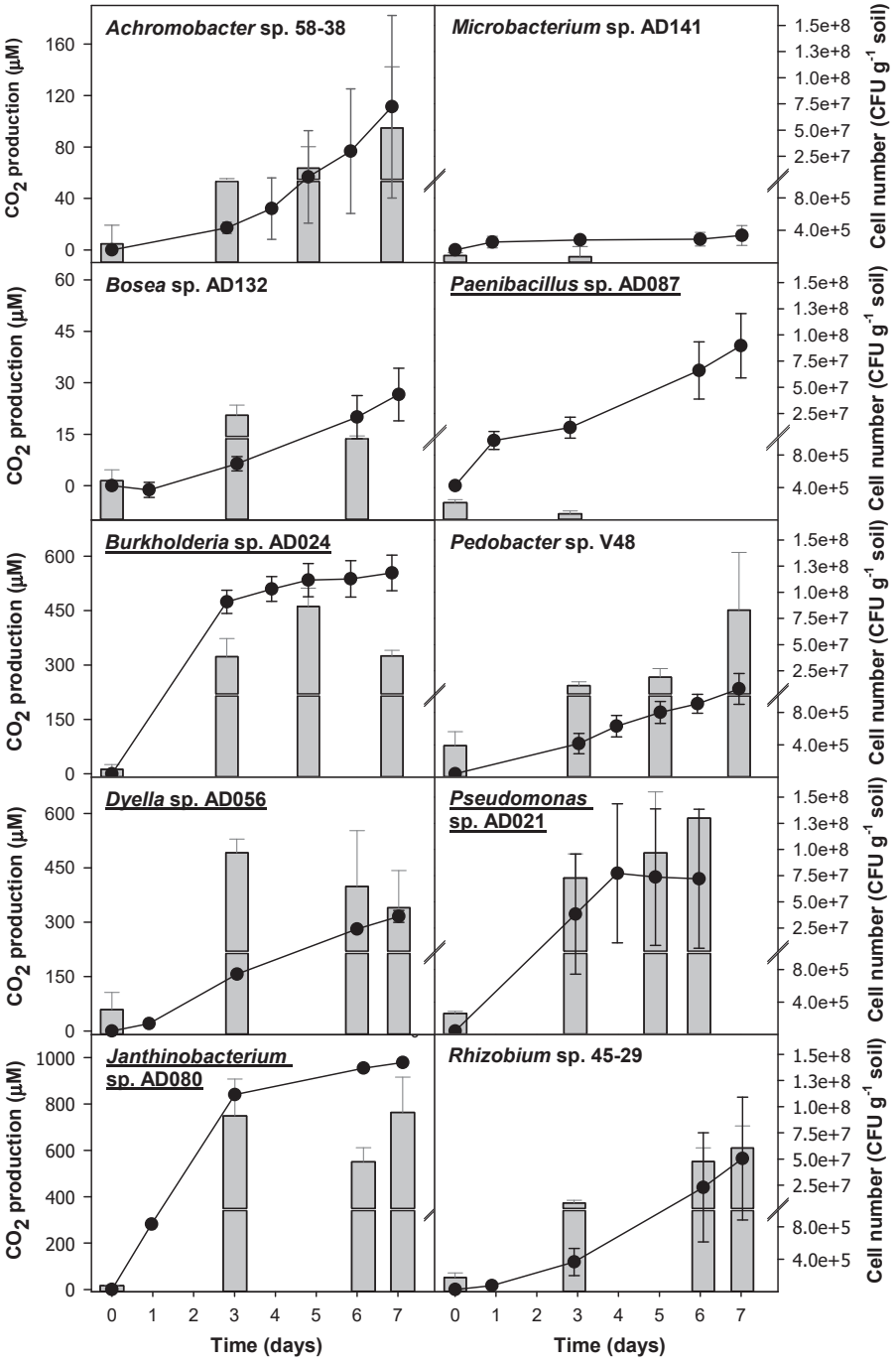

Figure S3.2. Growth (grey bars), represented by increase in Colony forming units (CFU) per g soil, and CO_2 production of bacteria (black dots) incubated in rhizospheric soil microcosms supplied with ARE. Data represent mean (n=3) and standard error. Bacterial strains selected for further analysis are underlined.

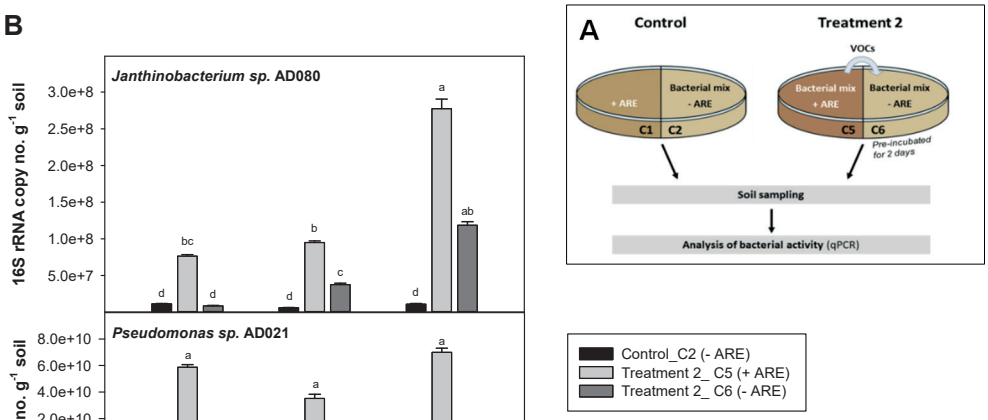

Figure S3.3. Influence of bacterial volatiles on nutrient-depleted bacteria. (B) 16S rRNA copy number per g soil were measured for a bacterial community of *Burkholderia sp*. AD024, *Dyella sp*. AD056, *Janthinobacterium sp*. AD080, *Pseudomonas sp*. AD021, and *Paenibacillus sp*. AD087 which was incubated in nutrient-poor soil. (A) In Treatment 2, bacteria in compartment C5 (dark grey) were pre-incubated for two days in nutrient-depleted soil before exposure to volatiles originating from bacteria supplied with artifi cial root exudates (ARE) in compartment C6 (light grey). The control compartment C2 (black) represents bacteria in nutrient-depleted soil not exposed to bacterial volatiles. Data represent mean (n = 9) and standard error corrected for the starting time point t0. Different letters indicate significant difference ($P<0.05$) between values resulted from one-way ANOVA and Tukey's HSD test.

CHAPTER 4

The Prey's Scent – Volatile Mediated Interactions between Soil Bacteria and their Protist Predators

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** Authors contributed equally to this work*

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Abstract

Protists are major predators of bacteria in soils, however, it remains unknown how protists sense their prey in this highly complex environment. Here, we investigated whether volatile organic compounds (VOCs) of six phylogenetic distinct soil bacteria affect the performance of three different soil protists and how that relates to direct feeding interactions. We observed that most bacteria affected protist activity by VOCs. However, the response of protists to the VOCs was strongly dependent on both, the bacterial and protist interacting partner. Stimulation of protist activity by volatiles and in direct trophic interaction assays often coincided suggesting that VOCs serve as signals for protists to sense suitable prey. Furthermore, bacterial terpene synthase mutants lost the ability to affect protists indicating that terpenes represent key components of VOCs-mediated communication. Overall, we demonstrate that volatiles are directly involved in protist-bacteria predator-prey interactions.

Keywords:

volatiles, bacteria-protist interactions, VOCs-mediated communication, terpenes

Soils are highly complex environments harboring an enormous diversity of organisms. Besides fungi, bacteria and protists are the most abundant and diverse soil organisms (Fierer & Jackson 2006; Geisen *et al.* 2015) that interact in many different ways. Protists are key predators of bacteria (De Ruiter *et al.* 1995; Bonkowski 2004) and they shape bacterial communities through selective feeding (Bonkowski & Brandt 2002; Rosenberg *et al.* 2009). Protists recognize prey quality when they are in direct contact through bacterial morphological differences and soluble compounds (Jousset 2012). However, it remains unknown whether protists sense their prey over long distances in the porous soil environment.

Due to their physico-chemical properties including low molecular weight, lipophilicity, high vapor pressure and low boiling points, volatile organic compounds (VOCs) can diffuse through air-and water-filled pores (Effmert *et al.* 2012). Consequently, they play a key role in interactions between physically separated soil microbes (Kai *et al.* 2009; Garbeva *et al.* 2014b; Schmidt *et al.* 2015; Schulz-Bohm *et al.* 2015). Most bacteria produce a broad spectrum of VOCs, which are of fundamental ecological importance in cross-kingdom interactions with e.g. plants, fungi, and nematodes (Gu *et al.* 2007; Kai *et al.* 2009; Effmert *et al.* 2012). However, whether protists as the most important predators of bacteria can sense bacterial VOCs and whether this information translates to prey suitability in specific bacteria-protist interactions remains largely unknown.

The responses of three different soil protists to VOCs emitted by six phylogenetically distinct soil bacteria were tested using a two-compartment Petri-dish system (Figure S4.1, Table S4.1 and S4.2). Our results revealed that bacterial VOCs significantly altered protist activity (Figure 4.1), i.e. higher relative abundance of trophozoites compared to cysts (Figure S4.1), as well as motility and growth (Figure S4.2 and S4.3), demonstrating that VOCs are key components in long distance communication between protist predators and bacterial prey. To evaluate whether VOCs-mediated responses reflect the outcomes of direct trophic interactions, we compared the impact of volatiles on protist activity with their responses in direct trophic interaction assays (Supplementary Material 4.1). In most cases, volatile-induced increases of protist activity were mirrored by an increase in activity in direct trophic interactions and *vice versa* (Figure 4.1). For example, the activity of *Vermamoeba* and *Saccamoeba* was reduced by *Dyella* whereas *Collimonas* stimulated the activity of *Vermamoeba* and *Tetramitus* in VOCs-mediated as well as in direct trophic interactions. This suggests that protists can sense suitable prey, based on bacterial species-specific VOCs. Specific long-distance diffusing bacterial VOCs can therefore provide early information about suitable prey and, consequently, more efficient predation.

However, opposing patterns were also observed. For instance, the presence of VOCs from *Burkholderia* and *Paenibacillus* reduced the activity of *Vermamoeba* and *Saccamoeba* whereas these protists increased in activity and abundance when directly preying on *Burkholderia* and *Paenibacillus* (Figure 4.1 and Figure S4.3). Similarly, VOCs of *Pseudomonas* stimulated activity of *Tetramitus* whereas the bacterium inhibited this protist in direct trophic interactions (Figure 4.1 and Figure S4.3). Opposing effects observed in VOCs-mediated and direct trophic interactions support the idea that bacteria and protist predators engage in a complex chemical warfare (Mazzola *et al.* 2009; Jousset 2012). Thereby besides producing soluble toxic compounds in presence of the predator (Mazzola *et al.* 2009; Pedersen *et al.* 2011; Jousset 2012), bacteria can repel potential predators by volatiles, which is in line with a report revealing inhibition of the protist *Acanthamoeba castellanii* by bacterial VOCs (Kai *et al.* 2009).

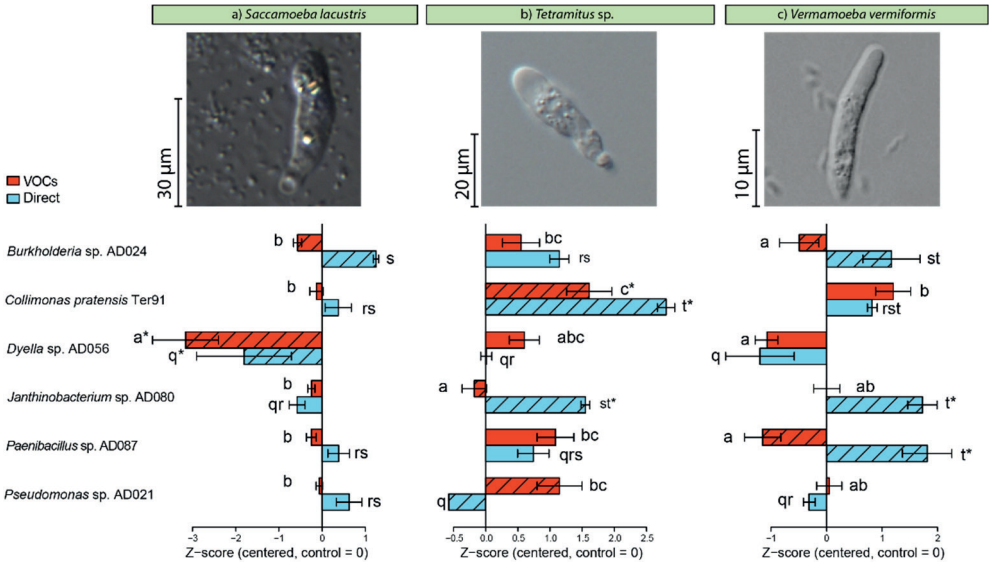

Figure 4.1. Effect (mean ± standard error) of six bacterial strains on the relative abundance of active protists (Figure S4.1) in three species (panels a, b and c) in volatile-mediated (red bars) and direct trophic (blue bars) interactions. Relative abundances were standardized (using Z-scores) per protist species and interaction type (direct vs. VOCs) and centred on the control treatment for each species. Positive values indicate more active protists relative to cysts than in the control (i.e., no bacterial volatiles or no bacteria in trophic interaction assays), negative values *vice versa*. Different letters indicate significant differences among bars tested per type of interaction (i.e. letters a-c for volatile-mediated interactions, and q-t for direct trophic interactions). Stars indicate significant differences from the control, while dashed bars indicate a significant difference between volatile-mediated and direct trophic interactions. For raw data see Figure S4.2 and S4.3, for statistical analyses Tables S4.3-4.5.

Similarly to bacterial-bacterial and bacterial-fungal VOCs-mediated interactions (Schmidt *et al.* 2015), the response of protists to bacterial volatiles was strongly dependent on the interacting partners (Figure 4.1 and Figure S4.2-S4.3). VOCs of *Burkholderia*, *Dyella* and *Paenibacillus* inhibited *Vermamoeba* and *Saccamoeba*, whereas they stimulated the activity of *Tetramitus*. The bacteria tested in this study produce distinct blends of volatiles (Garbeva *et al.* 2014b; Schulz-Bohm *et al.* 2015) that can explain the varying responses of the protist taxa. Species-specific bacterial-protist interactions are in line with differential feeding (Glücksman *et al.* 2010) and the sensitivity to soluble (toxic) bacterial secondary metabolites of protist taxa (Pedersen *et al.* 2011).

Bacterial volatiles belong to different chemical classes including alkenes, ketones, sulfides and terpenes (Lemfack *et al.* 2014; Schmidt *et al.* 2015), the latter group being especially large and diverse. Recently, Song *et al.* (2015b) showed that the *Collimonas* strain used in this study emits volatile monoterpenes (β-Linalool and β-Pinene) and sesquiterpenes (Germacrene D-4-ol and δ-Cadinene). To disentangle the contribution of specific groups of bacterial volatiles such as terpenes in bacterial-protist interactions, we created *Collimonas* strains mutated in the terpene synthase gene and phytoene synthase gene (Supplementary Material 4.1) and exposed protists to VOCs of those mutants. For the terpene synthase mutant, the activities of *Vermamoeba* and *Tetramitus* were similar to the control level and significantly reduced compared to the wildtype, demonstrating a loss of function in the mutant (Figure 4.2). Similar effects were observed for the motility of *Vermamoeba* and *Tetramitus* (Figure S4.4). VOCs of the phytoene synthase mutant did not significantly change the activity of *Vermamoeba* and *Tetramitus* compared to the wildtype (Figure 4.2). These results suggest that terpenes play a key role in VOCs-mediated bacteria-protist interactions as catalysers of protist activity which is in line with terpene-induced stimulations of nematodes (Rasmann *et al.* 2005). To date the ecological and the biological function of bacterial terpenes remains largely unknown. Most terpenes volatilize easily and thus can travel fast and over long distances through both the liquid and gaseous phase of the soil (Hiltpold & Turlings 2008). Hence, terpenes may be of great ecological importance for the interactions between spatially distant soil organisms.

In conclusion, we show that volatiles are key drivers of species-specific bacterial-protist-interactions and that terpenes are among the informative compounds that enable protists to sense suitable prey bacteria. Species-specific responses of protists to bacterial VOCs suggest potential co-evolutionary dynamics in predator-prey interactions. Our study further suggests that specific volatiles can be used to activate protist predators of soil-borne disease agents, which might serve as a new method for biocontrol. Further work should aim

at investigating the mechanisms and importance of volatile-mediated interactions in more complex settings, including natural conditions in soils.

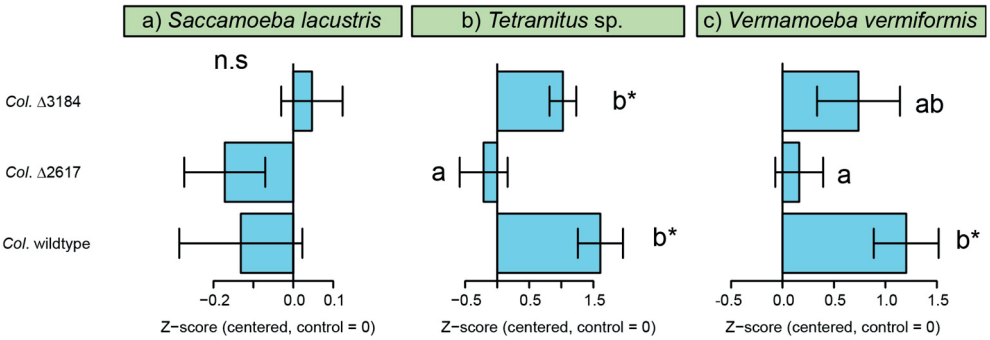

Figure 4.2. Effect (mean ± standard error) of volatiles of wildtype *Collimonas pratensis* Ter91 and two mutants (Δ2617: no terpene synthase activity; Δ3184: no phytoene synthase activity) on the activity of three protist species (panels a, b and c). Phytoene synthase mutant (*Col.* Δ3184) and terpene synthase mutant (*Col.* Δ2617) prevent production of non-volatile and volatile terpenes, respectively. Protist activity was standardized (using Z-scores) per protist species and centred on the control treatment for each species. Positive values indicate higher protist activity than in the control (i.e., no bacterial volatiles), negative values *vice versa*. Different letters indicate significant differences among bars. Stars indicate significant differences from the control. For raw data see Figure S4.4, for statistical analyses Tables S4.6.

Acknowledgements

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Author Contributions

KS and SG designed and performed the experiments. CS constructed the *Collimonas* mutants. KS, SG and ERJW analyzed and interpreted the data, KS and SG wrote the paper with contributions from all co-authors.

Supplementary Material 4.1 (Material and Methods)

Bacterial strains, protists and growth conditions

Bacterial strains were previously isolated from different dune soil sites (De Boer *et al.* 1998; De Boer *et al.* 2004; De Ridder-Duine *et al.* 2005) (Table S4.1) and cultured as described before (Schulz-Bohm *et al.* 2015). Monoxenic protist cultures (Table S4.2) were obtained from an enrichment cultivation approach as described in Geisen *et al.* (2014).

Table S4.1. Bacterial strains used in this study. All strains are from organic poor, sandy dune soils in the Netherlands.

Bacterial strain (Accession number)	Origin	Phylum *(family)*
Collimonas pratensis Ter91	Inner coastal dune soil in Terschelling (*De Boer et al. 1998; De Boer et al. 2004)*	*Beta-Proteobacteria (Oxalobacteraceae)*
Burkholderia sp. AD024 (KJ685239)	Dune grassland near Ouddorp, Zeeland *(De Ridder-Duine et al. 2005)*	*Beta-Proteobacteria (Burkholderiaceae)*
Dyella sp. AD056 (KJ685269)	Drift sand near Loon op Zand, Brabant *(De Ridder-Duine et al. 2005)*	*Gamma-Proteobacteria* (*Xanthomonadaceae*)
Janthinobacterium sp. AD080 (KJ685292)	Coastal outer dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	*Beta-Proteobacteria (Oxalobacteraceae)*
Paenibacillus sp. AD087 (KJ685299)	Pine plantation near Loon op Zand, Brabant *(De Ridder-Duine et al. 2005)*	*Firmicutes* (Paenibacillaceae)
Pseudomonas sp. AD021 (DQ778036)	Coastel inner dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	*Gamma-Proteobacteria (Pseudomonadaceae)*

Table S4.2. Protist species used in this study. All protists were isolated from sandy soil in the Millingerwaard (the Netherlands).

Protist strain	Eukaryotic supergroup	Class	Order	Family
Vermamoeba vermiformis	*Amoebozoa*	*Tubulinea*	*Echinamoebida*	*Vermamoebidae*
Saccamoeba sp.	*Amoebozoa*	*Tubulinea*	*Euamoebida*	*Hartmannellidae*
Tetramitus sp.	*Excavata*	*Heterolobosea*	*Schizopyrenida*	*Vahlkampfiidae*

In frame deletion of the terpene synthase gene (CPter91_2617) and phytoene synthase gene (CPter91_3184) in *Collimonas pratensis* Ter91

In frame deletion of the terpene synthase gene (CPter91_2617) and phytoene synthase gene (CPter91_3184) was performed with the pEX18Tc suicide vector as described by Choi and Schweizer (2005). For each mutant construct, 804 bp and 808 bp upstream fragments before the 5′-end and 807 bp and 809 bp downstream fragments after the 3′-end for terpene synthase gene and phytoene synthase gene, respectively, were synthesized by Baseclear, Leiden, the Netherlands (www.baseclear.com), and constructed into the vector pEX18Tc. The synthesized sequences are given in the end of this file. For the terpene synthase gene, EcoRI and HindIII were designed for constructing with pEX18Tc; while for the phytoene synthase gene, EcoRI and XbaI were designed for constructing with pEX18Tc. The mutant constructs pEX18Tc-2617 and pEX18Tc-3184 were subsequently electroporated into *C. pratensis* Ter91. Electrocompetent cells were obtained according to the method of Choi *et al.* (2006) and the electroporation was performed at 2.4 kV and 200 μF. After the incubation in SOC medium (2% Bacto tryptone [Difco], 0.5% Bacto yeast extract [Difco], 10 mM NaCl, 2.5 mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose [pH 7]) for 2 h at 25°C, the cells were plated on KB supplemented with tetracycline (25μg/ml). The obtained single crossover colonies were grown in LB overnight at 25°C and plated on LB supplemented with 5% sucrose to accomplish the double crossover. The plates were incubated at 25°C for at least 48 h, and colonies were re-streaked on LB supplemented with tetracycline (25μg/ml) and on LB supplemented with 5% sucrose. Colonies that grew on LB with sucrose, but not on LB with tetracycline, were selected and subjected to colony PCR to confirm the deletion of the genes. Primers were CPter91_2617_deletion check primer_F: TCAAGCAAGCAAGCTTCGCCGACCTGAC GCAAAAGC; CPter91_2617_deletion check primer_R: TCAAGCAAGCAAGCTTGCT AGCCTGAGCTGGGCCTT; CPter91_3184_deletion check primer_F: AAGTGGCATAAG CAGTCTGA; CPter91_3184_deletion check primer_R: CAGGAACAGGAAGCTGTAGT

Synthesized sequences for the terpene synthase gene (CPter91_2617) and phytoene synthase gene (CPter91_3184).

Sequences in black represent the upstream fragments before 5'-end of the target genes. Sequences in grey represent the downstream fragments after the 3'-end of the target genes.
In frame deletion of terpene synthase gene CPter91_2617 in Ter91 (EcoRI and HindIII sites)

GAATTCtgaatcgcttccatctggccggccacccggagatcctgatcgtttccaatgtcatcgaagaaggcaagccgatcggcctg
gtggatgccggcgccgactggcattccgacctgtcctacatgcccaggcccagcctgggctcgctgctgcatgcgcaagaattacc
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gaaatagcgcgccggcgtaaacggcatggcggccacttctaccggcaccgccttaccgcgcaccagtgcatgcaattgggtgccg
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gcccagctcaggctagcctcgactggcgtggtggagacatccatgtcgtgcccgtacaggcacagaccggcttccagacgcagag
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ggagatttcaaaaccgtcttcgccggtatagccggaacggctgatgaaacattcagcgcccgccagcgtgactttgccggtttgcat
gaagaccatttgcgcggtttccggcgccaggcgcgccatcaccgcagcggccgccggtccttgcaaagccagcagggaacgatc
gcccaactcctcgatctggcaacggccggccaggtgctggcgcagatgcgcggtatcttgctgcttgcaggcaAAGCTT

In frame deletion of phytoene synthase gene CPter91_3184 in Ter91 (EcoRI and XbaI sites)

GAATTCaagtggcataagcagtctgattcaactgcccttgaaagccgcctcggcctggtcataggtcgcttgcgccgccttgaag
gcggtgtccttggcgtccagcaccgcctggctgacgaagttcttgccgcgcagctcctgatagcgtttcaagtcggccttggccgtat
cgcggttgctggcggcggaactcaccgccgccaccgcttgcgcttgcgccagctgcaaatccttggggtccagccgcatcagcacc
tggccgcgcttgaccaccgtgccaacatccacctggcgggcgatgatcttgccccccacccggaagcccagctgcgattcgtagcg
cgcctgcactgcaccgggaagctcggaggtgacgtcggcattggccggattgagcactgtcacgcgcaccgggcggatatcctcg
acctttccggcgccttggaacaggccgccagcagggtcaatgtacaaacaagcaataaattgggagcacgcatcaagccgtgga
gaggggctgggtcgcgctcgattgcacgctgaattatctgggtcggcacgttttttcctttactattctgggctattgccagtttgcgcat
tgccatcaggatacgtagtgatcacggcgggctgctgcagcttccatcccgccctcaaaaattactaactctaaagtcagtaattataa
ttgcgagcgatcaagtttgcccaaggctttttgcgtccgccagcaattgccacaaacatggctttttaacaaatgttttacacagcggtt
ttacacagcggttttacacagcgccccaaaaatcgataaaaatcagcaagcaagcagcatttcttacgtatacttcgggcaagtcgtt
ttcaagctgtccgtagccgccagcaccaagtattcattagcgctcattaccgttttttatatccatgtcaccagacgattattgccagcaa
aaagccgcccaaagcggttccagttttactacagcttcctgttcctgccggtcgagcgtcgccgcgccattactgccctgtatgcgtttt
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gccgccatgctggccggtaatccctcgcatccggtgacccaggcgctgcagccgcacctgctgacctatggactggaaggcaagc
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tcgccttccagctcaccaacatcatccgcgacgtcggcgaagatggccgcaagggtcgtatttatttaccggttaacgaactgcagca
attcaatgtcaccgcggccgatatcctcaatgcccgctacagcgagaatttcgtcaacctgatgcgcttccaggtggcgcgcgcaca
gcaagcttacgatgaagcgtttgccttgctgcccaagcaggaccggcgTCTAGA

Exposure of protists to bacterial volatiles

Two-compartment Petri-dishes were filled with 12 ml 1.5 % (v/v) water-agar (WA) supplied with artificial root exudates (18 ml artificial root exudates stock solution [Schulz-Bohm *et al.* 2015] per liter WA [Schmidt *et al.* 2016]) on one side and 12ml 0.5 % (v/v) water-agar without phosphate (5 g agar [Merck, The Netherlands] in 1l Demi-water, pH 6.5) on the other side (Figure S4.1). Protists were washed with sterile phosphate-buffer (10 mM KH_2PO_4, pH 6.5) and mixed with three different antibiotics (5 mg ml^{-1} ampicillin, 0.4 mg ml^{-1} rifampicin, and 0.5 mg ml^{-1} kanamycin) to inhibit the growth of associated and co-transferred bacteria. A droplet of 20 µl protist-antibiotic solution was place in the middle of the one compartment filled with WA and incubated overnight at room-temperature. Bacterial solutions of 10^8 CFU ml^{-1} per strain were prepared (Schulz-Bohm *et al.* 2015) and 50 µl of the solution was spread on the agar in the other compartment (Figure S4.1). Petri-dishes (six per treatment) were sealed with parafilm and incubated for six days at 20°C. Cysts and trophozoites (i.e. active stage of the protists) and migrated protist individuals (Figure S4.1) were counted under an inverted Leica DMIL microscope (Germany) with a Leica C Plan L20x/0.30 or L40x/0.50 PH2 objective.

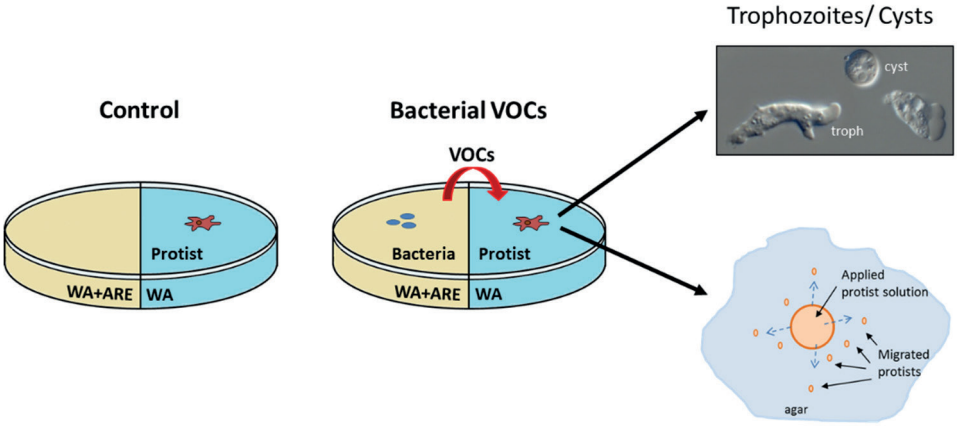

Figure S4.1. Experimental setup to assess the effect of bacterial volatiles on protists. Protist activity was expressed by the relative abundance of trophozoites from the total number of protists (i.e. including encysted individuals). Potential induced motility of the protists by exposure to volatiles was determined by counting migrated protists outside the spot where the protist solution was initially placed (big orange circle).

Direct trophic interaction assay

In 24-well Cellstar® suspension culture plates (Greiner bio-one, Germany), 20 µl of washed and antibiotic treated protist-solution per well was mixed with 100 µl of bacterial suspension (10^8 CFU ml^{-1}) or sterile phosphate-buffer (control) and incubated at 20°C. The total number of protist as well as the number of cysts and trophozoites was determined with an inverted Leica DMIL microscope (Germany) with a Leica C Plan L20x/0.30 or L40x/0.50 PH2 objective at the beginning and after days of incubation.

Statistics

The data for the sub experiments were analysed separately using generalized linear models (GLMs). In each analysis protist species identity, bacterial treatment and their interaction were included as factors. However, as differences among protist species were not the focus of our study their main effect was not tested for significance. Relative abundance data of trophs to cysts were standardized (z-transformation) per protist species and analysed using GLMs with Gaussian error structure. Migration and total abundance data were modelled as count data with overdispersion using negative binomial GLMs (O'Hara & Kotze 2010).

To compare the effects of bacterial volatiles with the effect of direct trophic interaction with the same bacterial strain we combined the relative abundance of active protists data of both sub experiments. We took the z-transformed relative abundance data of both sub experiments and centred them on the mean relative abundance of the respective controls per protist species (i.e. relative abundance in control: Z-score = 0). We tested for differences in responses using a GLM with Gaussian errors and compared the differences in response among direct and volatile-mediated interactions per protist species and bacterial treatment only using planned contrasts to reduce the number of comparisons.

All analyses were conducted in R version 3.2.4 Team 2013, negative binomial GLMs were done in package MASS version 7.3-45 (Venables & Ripley 2013), and multiple comparisons in package lsmeans version 2.20-23 (Lenth 2016). Model assumptions were checked using residual plots.

Supplementary Material 4.2 (Supporting Results)

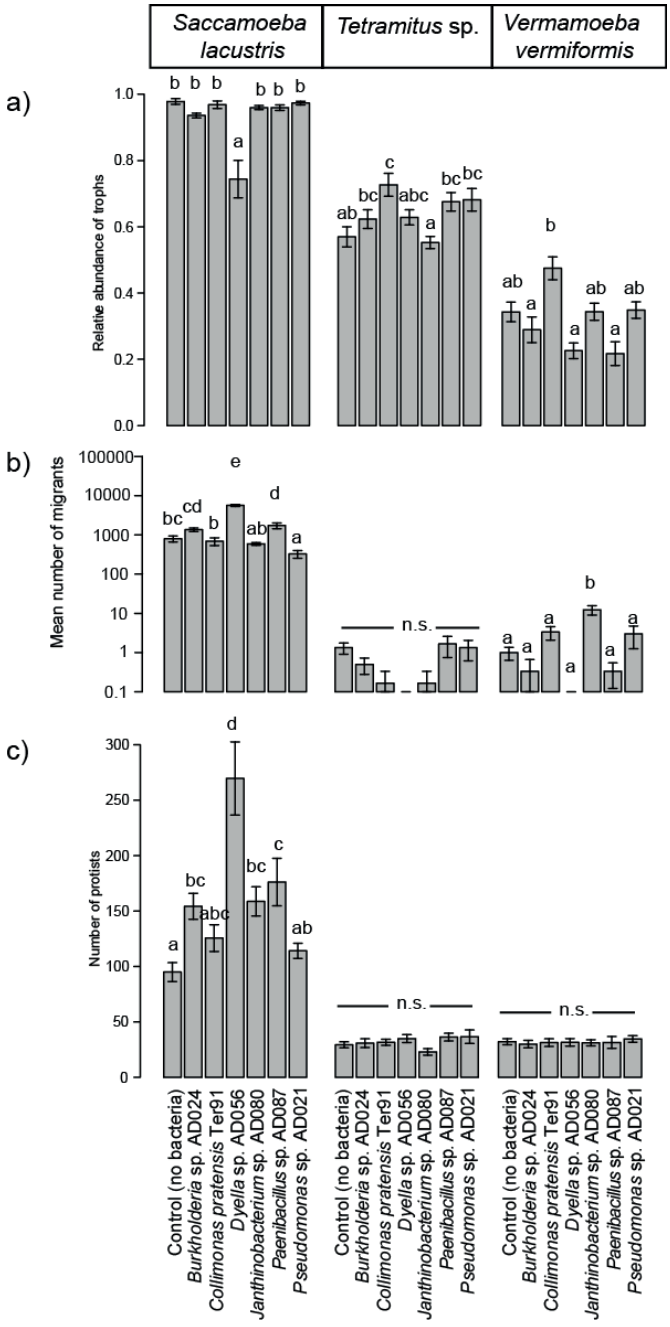

Figure S4.2. Raw data for the volatile-mediated bacteria-protist interaction experiments. The relative abundance of active protists, i.e. trophozoites (a), migration (b), and total numbers of protists (c) was analysed. Shown are volatile-mediated responses (mean ± SE) of three protist species to six bacterial strains and a control where no bacteria were added to the other compartment. High density of protists in Dyella-Saccamoeba interaction probably caused self-inhibition resulting in a lower relative abundance of trophozoites compared to the control. Different letters indicate significant differences among bars tested per protist species. n.s. = not significant. See Table S4.3 for results of the overall analyses.

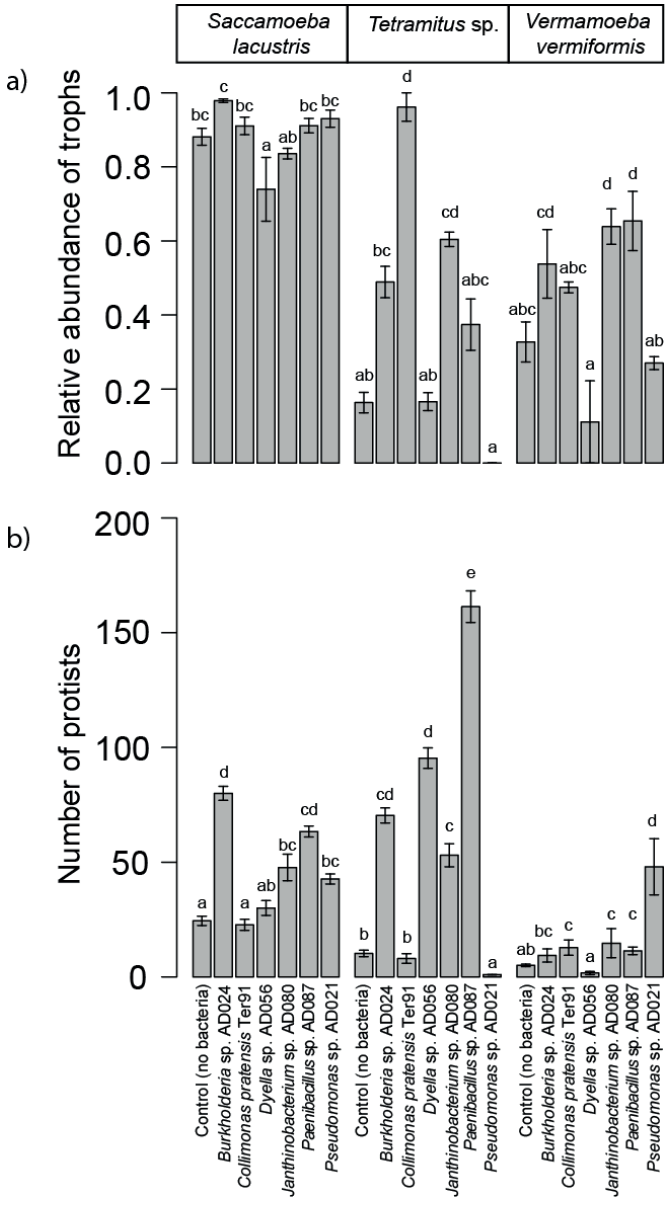

Figure S4.3. Raw data for the direct interaction experiments for relative abundance of active protists, i.e. trophozoites (a) and total numbers of protists (b). Shown are direct trophic interaction responses (mean ± SE) of three protist species to six bacterial strains and a control where no bacteria were added. Different letters indicate significant differences among bars tested per protist species. n.s. = not significant. See Table S4.4 for results of the overall analyses.

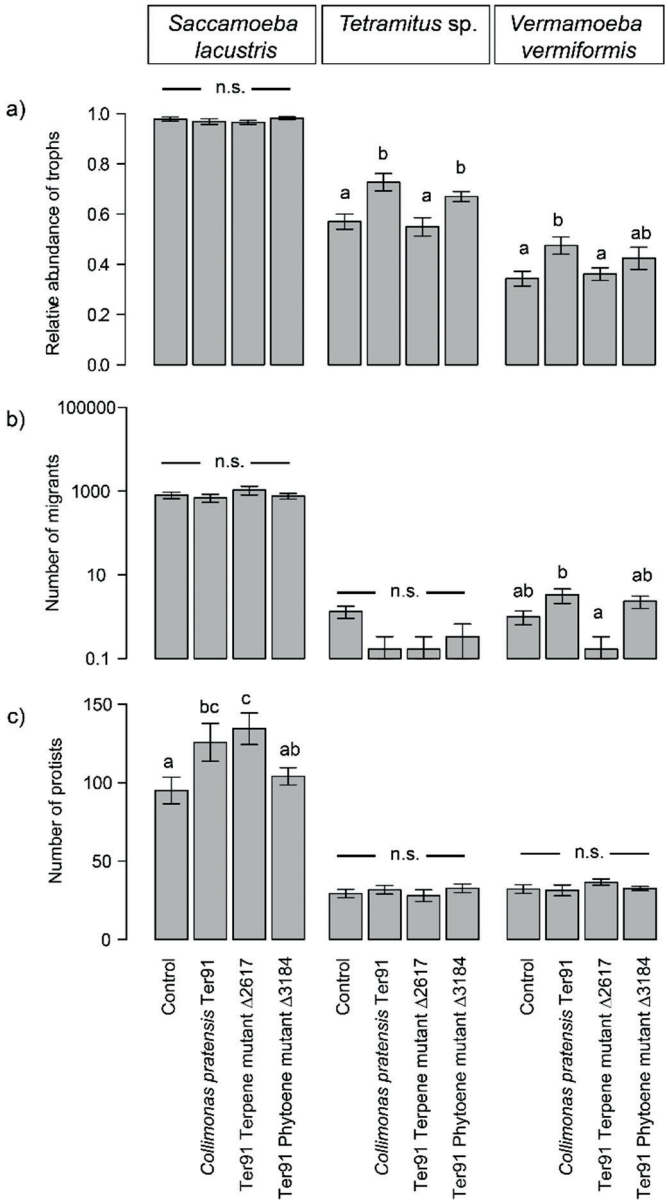

Figure S4.4. Raw data for the volatile-mediated interaction experiments between the wildtype and two terpene mutants (Δ2617: no terpene synthase activity; Δ3184: no phytoene synthase activity) of Collimonas pratensis Ter 91. For the control, no bacteria were added to the other compartment. The relative abundance of active protists, i.e. trophozoites (a), migration (b), and total numbers of protists (c) was analysed. Different letters indicate significant differences among bars tested per protist species. n.s. = not significant. See Table S4.3 for results of the overall analyses.

Table S4.3. Summary of the statistical analyses of the VOC experiment. Although an effect of protist species was included in the models it was not tested for significance as this was not the focus of our study. LRT = likelihood ratio test statistic.

	Troph relative abundance*			Migration			Protist abundance		
Term	d.f.	F	P	d.f.	LRT	P	d.f.	LRT	P
Protist	-	-	-	-	-	-	-	-	-
Bacteria	6, 104	13.97	<0.001	6	28.98	<0.001	6	31.88	<0.001
P x B	12, 104	6.41	<0.001	12	213.38	<0.001	12	59.01	<0.001

* Response variable was z-transformed.

Table S4.4. Summary of the statistical analyses of the direct trophic interaction experiment. Although an effect of protist species was included in the models it was not tested for significance as this was not the focus of our study. LRT = likelihood ratio test statistic.

	Troph relative abundance*			Protist abundance		
Term	d.f.	F	P	d.f.	LRT	P
Protist	-	-	-	-	-	-
Bacteria	6,51	19.61	<0.001	6	32.50	<0.001
P x B	12,51	5.89	<0.001	12	767.12	<0.001

* Response variable was z-transformed.

Table S4.5. Summary of the statistical analysis of the effects of interaction type (volatile mediated or direct trophic interactions) on the relative abundance (Z-scores) of three protist species in response to six bacterial strains. Z-scores were centred per protist species on the control (i.e. control Z-score = 0).

Term	d.f.	F	P
Protist	2	-	-
Interaction type	1	28.89	<0.001
Bacteria	5	27.42	<0.001
P x I	2	3.15	0.046
P x B	10	5.98	<0.001
I x B	5	5.79	<0.001
P x I x B	10	5.71	<0.001
Residual	128		

Table S4.6. Summary of the statistical analyses of the terpene synthesis mutant experiment. Although an effect of protist species was included in the models it was not tested for significance as this was not the focus of our study. LRT = likelihood ratio test statistic.

	Troph relative abundance*			Migration			Protist abundance		
Term	d.f.	F	P	d.f.	LRT	P	d.f.	LRT	P
Protist	-	-	-	-	-	-	-	-	-
Bacteria	3,60	9.39	<0.001	3	0.03	0.99	3	8.17	0.04
P x B	6,60	2.79	0.019	6	16.21	0.013	6	11.39	0.08

* Response variable was z-transformed.

CHAPTER 5

Fungus-Associated Bacteriome in Charge of their Host Behavior

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Abstract

Bacterial-fungal interactions are widespread in nature and there is a growing number of studies reporting distinct fungus-associated bacteria. However, little is known so far about how shifts in the fungus-associated bacteriome will affect the fungal host's lifestyle. In the present study, we describe for the first time the bacterial community associated with the saprotrophic fungus *Mucor hiemalis,* commonly found in soil and rhizosphere. Two broad-spectrum antibiotics that strongly altered the bacterial community associated with the fungus were applied. Our results revealed that the antibiotic treatment did not significantly reduce the amount of bacteria associated to the fungus but rather changed the community composition by shifting from initially dominating *Alpha-Proteobacteria* to dominance of *Gamma-Proteobacteria*. A novel approach was applied for the isolation of fungal-associated bacteria, which also revealed differences between bacterial isolates obtained from the original and the antibiotic-treated *M. hiemalis*. The shift in the composition of the fungal-associated bacterial community led to significantly reduced fungal growth, changes in fungal morphology, behavior and secondary-metabolites production. Furthermore, our results showed that the antibiotic-treated isolate was more attractive and susceptible to mycophagous bacteria as compared to the original isolate. Overall, our study highlights the importance of the fungus-associated bacteriome for the host's lifestyle and interactions and indicate that isolation with antibacterials is not sufficient to eradicate the associated bacteria.

Keywords:

Mucor hiemalis, fungal bacteriome, fungal behavior, volatiles, interactions

Introduction

Bacteria and fungi are widespread in nature and play important roles in many ecological processes. Similar to other organisms, many fungi have an associated bacteriome and there is a growing number of known endosymbionts where bacteria dwell within fungal hyphae (Bonfante & Anca 2009; Kobayashi & Crouch 2009). Another type of fungal-bacterial interaction is ectosymbiosis, where the bacterial partner is adhered to fungal hyphae (Warmink *et al.* 2009; Stopnisek *et al.* 2016).

The symbioses with ecto- and endofungal bacteria are often overlooked, yet they may have a profound effect on the fungus behavior and lifestyle. For example, in *Aspergillus nidulans* and *A. niger*, the ectobacteria actinomycetes and *Bacillus subtillus* respectively, were shown to affect fungal primary and secondary metabolism (Schroeckh *et al.* 2009; Benoit *et al.* 2015).

It is plausible that there is a relationship between fungi and bacteria of which both partner profit. For example, bacteria or fungi can benefit from specific compounds that are produced by the other partner if they cannot produce it themselves. Several mycorrhizal helper bacteria secrete citric and malic acids that are metabolized by *Laccaria bicolor*, promoting its growth (Duponnois & Garbaye 1990). Conversely, ectomycorrhizal fungi may produce organic acids or sugars that can affect the composition and growth of associated bacterial communities. For example, the helper bacterial isolate *P. fluorescens* BBc6R8 can be chemoattracted by the hyphae of the ectomycorrhizal fungus *L. bicolor* S238N and the trehalose accumulated within the mycelium that promots the growth of the helper bacterium (Deveau *et al.* 2010). In the interaction between *S. cerevisiae* and several *Acinetobacter* species, ethanol secreted by the yeast was shown to stimulate the growth of the bacterial species and it can act as a signaling molecule, altering cell physiology (Smith *et al.* 2004).

Bacterial endosymbionts *Candidatus Glomeribacter gigasporarum* of arbuscular mycorrhizal fungi *Gigaspora margarita* can be involved in the vitamin B12 provision for the fungus (Ghignone *et al.* 2012). The co-adaptation between fungal hosts and bacterial endosymbionts can become so tight that the genome size and the gene content of the endosymbionts is reduced. Mycorrhizal endosymbionts from Mollicutes lineages were suggested to depend metabolically on their host, and additionally have taken up regulatory eukaryotic genes horizontally (Naito *et al.* 2015). Clear evidence for gene transfer between Mollicute-related endobacteria and their mycorrihizal host *Dentiscutata heterogamma* was recently reported (Torres-Cortes *et al.* 2015). Furthermore, endobacteria can improve the fitness of their host by e.g. increasing the fungal sporulation success and raising the fungal bioenergetics capacity (Salvioli *et al.* 2016). Another well-studied example is the rice seedling

blight pathogen *Rhizopus microsporus* (Lackner *et al.* 2009; Partida-Martinez & Hertweck 2005). This fungus contains endobacteria named *Burkholderia rhizoxinica* and *Burkholderia endofungorum* (Partida-Martinez *et al.* 2007a), which have been shown to produce a potent toxin involved in host pathogenesis (Partida-Martinez & Hertweck 2005; Gee *et al.* 2011). Interestingly, the endobacteria enforce their vertical transmission by controlling host sporulation making use of a hrp type III secretion system (Partida-Martinez *et al.* 2007b; Lackner *et al.* 2011). As a result, the host is not able to reproduce in absence of its endosymbiont, thereby ensuring maintenance of the symbiosis (Partida-Martinez *et al.* 2007b).

Microscopic and molecular analysis showed that several nitrous oxide-producing fungal isolates of *Mortierella elongata* harboured endobacteria in their mycelia (Sato *et al.* 2010). The sequencing of 16S rRNA genes revealed that the N_2O-producing fungus *Mortierella elongate* harbored endobacteria belonging to the family *Burkholderiacea,* however, the significance of this fungal-bacterial association is unknown (Sato *et al.* 2010). Recently, an endophyphal bacterium living in association with *Mortierella elongate* was isolated and on the basis of phenotypic, chemotaxonomic and pylogenetic characteristics it was identified as a novel genus and species, for which the name *Mycoavidus cysteinexigens* gen. nov., *sp.*, nov was proposed (Ohshima *et al.* 2016).

It is a common practice prior performing experiments with soil-borne fungi to pre-culture them on a media supplemented with antibiotics (Ballhausen *et al.* 2016; Singh *et al.* 2015). In our lab working with the saprotrophic fungus *Mucor hiemalis* (Zygomycota) isolated from the rhizosphere of *Carex arenaria* (sand sedge) (De Rooij-van der Goes *et al.* 1995), we observed that treatment with broad-spectrum antibiotics strongly altered the fungal morphology and hyphal extension. Based on this observation, we aimed first to determine the bacterial community associated with *Mucor hiemalis* and to test if the antibiotic treatment resulted in bacteria-free fungus. Furthermore, we aimed to reveal how changes in the bacterial community affect fungal fitness, behavior, metabolites production and interactions.

Material and Methods

Fungal strains and growth conditions

The *Mucor hiemalis* isolate M0 obtained from the rhizosphere of *Carex arenaria* (Sand sedge) collected from sandy dune soil in the Netherlands was originally isolated on malt extract agar supplemented with 50 ppm oxytetracycline (De Rooij-van der Goes *et al.* 1995). Here, the antibiotic-treated isolate MA derived from the isolate M0, after plating on King's B agar with antibiotics (rifampicin and kanamycin 50 mg/ml, final concentration), and transferred to Water Yeast Agar (WYA) (Garbeva *et al.* 2011b) and Oatmeal agar OA (24 g/L Difco, France) supplemented with rifampicin and kanamycin (50 mg/ml final concentration). The spores were washed in rifampicin and kanamycin solution (50 mg/ml final concentration) and collected over glass wool with sterile deionized water and stored at -80°C. Spores of the M0 and MA isolates were grown on nutrient-rich 0.5 strength Potato dextrose agar (PDA, Oxoid, England; pH 6), and nutrient-poor Water agar + $(NH_4)_2SO_4$, pH 6.7 (Garbeva *et al.* 2011b; Garbeva *et al.* 2014b).

Fungal identification

The identity of the isolate M0 and MA was confirmed using the ITS sequences targeted by the primers ITS1 and ITS4 (White *et al.* 1990). Fungal DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, USA) according to the manufacturer's instructions. Extracted DNA was used for PCR amplification with a master mix containing 1x FastStart High Fidelity Reaction Buffer (Roche) with 18 mM $MgCl_2$ (Roche), 0.04 U FastStart High Fidelity Enzyme Blend (Roche), 200 µM of each dNTP, 0.6 µM ITS1 and ITS4 primer. The thermal protocol was as follows: initial denaturation at 95°C for 5 min, and 35 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, and a final elongation at 72°C for 10 min. The PCR product was cleaned using a PCR purification kit (QIAGEN Benelux B.V., The Netherlands) and sent for Sanger sequencing to Macrogen Europe (Amsterdam, The Netherlands). Obtained sequences were checked for quality using BioEdit (Hall 1999) and identified using NCBI nucleotide database BLASTn (Altschul *et al.* 1990).

Hyphal extension of fungal isolates M0 and MA

For measuring hyphal extension, 6-mm-diameter agar disks taken from the edge of the fungal hyphae of M0 or MA (pre-grown on 0.5 strength PDA) were plated in the middle of nutrient-rich 0.5 strength PDA or nutrient-poor WA plates (8.5 cm diameter). Per fungal isolate and agar type (i.e. PDA and WA) three plates were set up. The plates were sealed with parafilm and incubated at 20 °C for 5 days. On the fifth day, for each plate the extension

of fungal hyphae was measured with a ruler in three coordinates and the average extension was calculates.

Competition assay of fungal isolates M0 and MA

Two plugs taken from the edge of the (on 0.5 strength PDA) pre-grown fungal hyphae of M0 or MA were placed with 3 cm distance from each other on a new 0.5 strength PDA plate (8.5 cm diameter) and incubated at 20°C. As control, both fungal isolates were incubated separately on 0.5 strength PDA at the same time. For each treatment three replicates were set up. The hyphal extension was monitored at day 7, 14 and 21 after inoculation. Pictures were taken with a Panasonic DMC-FZ200 digital camera. The area of M0 and MA hyphal extension was measured with AxioVision V 4.9.1.0 (Carl Zeiss Microscopy GmbH, Germany). A relative area for the hyphal extension per replicate was calculated by dividing the area obtained in the competition experiment per fungal isolate by the average area obtained for each control.

Bioassay for testing the fungal growth inhibition by bacterial secondary metabolites

The bacterial strains *Burkholderia sp.* AD024 (De Ridder-Duine *et al.* 2005; Schulz-Bohm *et al.* 2015) and *Collimonas fungivorans* Ter 331 (De Boer *et al.* 2004) were incubated overnight at 22°C in 0.1 Tryptic Soya Broth (TSB) (Tyc *et al.* 2014), washed with phosphate buffer (10mM KH_2PO_4, pH 6.5), and the OD_{600} was adjusted to 10^8 cells per ml. 50 µl of the bacterial suspension was spread horizontally in the middle of a Petri-dish containing 1.5 % (v/v) water agar (Schmidt *et al.* 2016) supplied with artificial root exudates (WA + ARE) as described previously by Schulz-Bohm *et al.* (2015). Per liter WA, 18 ml ARE stock solution was added, implying that 134 µg carbon per ml agar was additionally supplied. Bacteria were incubated for three days at 20°C and a plug of 8 mm diameter with fungal hyphae (pre-grown on 0.5 strength PDA) was transferred to the top of the Petri-dish. Agar plates were wrapped with two layer of parafilm and incubated at 20°C. After four days, the distances between the edge of the fungal hyphae and bacterial biofilm (refer to as inhibition zone) were measured. Pictures of agar plates were taken with a Panasonic DMC-FZ200 digital camera. Pictures of fungal hyphae were taken and processed with a Olympus SZX12-ILLK200 stereo microscope (Olympus Cooperations, Japan) in combination with AxioVision V 4.9.1.0 (Carl Zeiss Microscopy GmbH, Germany).

Bacterial and yeast isolation and identification

Mycelium of M0 and MA (pre-grown on 0.5 strenght PDA) was collected in 7 ml sterile phosphate buffer (10 mM KH_2PO_4, pH 6.5). The mycelium was sheared using a MICRA D-9 homogenizer (MICCRA GmbH, Germany) for 5 minutes. The resulting suspension was shaken for 45 min at 22°C and filtrated using membrane pore sizes of 10µM (Millipore, The Netherlands) as well as 3µM (Schleicher and Schuell, Germany). 350 µL aliquots of the filtrate were plated on R2A medium (Difco, France), WYA and 0.1 TSB (Tyc *et al.* 2014) containing 100 mg/L filter sterilized cycloheximide (Sigma-Aldrich, The Netherlands) and 50 mg/L thiabendazole (Sigma-Aldrich, The Netherlands) to inhibit the growth of M0 and MA. Three volatile compounds: Dimethyldisulfide, Dimethyltrisulfide and Benzonitrile (Sigma-Aldrich, The Netherlands) with known antifungal but no antibacterial activity (Garbeva *et al.* 2014a), were added in concentrations of 160 µmol to a sterile filter paper positioned at the edge of the plate as described previously by Garbeva *et al.* (2014a). Plates supplemented with volatiles were kept in a protective cabinet at room temperature (± 20°C).

PCR amplification of 16S rRNA genes from the isolates was performed either directly with colony material diluted in sterile deionized water or with isolated DNA using the QIAamp DNA Mini Kit (Qiagen, The Netherlands) according to the manufacturer's protocol. The PCR mix contained 0.17 mg/ml BSA, 0.33 µM of each primer (27F or 515F and 1492r; Lane 1991), 1.5-2 µl template, and 1x PCR Mastermix (Thermo Scientific, The Netherlands) containing 0.05U/µl Taq DNA polymerase, reaction buffer, 4 mM $MgCl_2$,and 0.4 mM of each dNTP. The thermal protocol was as followed: initial denaturation at 95°C for 8 min, 5 cycles at 95°C for 60 s, at 40°C for 60 s, and at 72°C for 90 s, and 35 subsequent cycles at 95°C for 60 s, at 53°C for 30 s, and 72°C for 70 s. The final elongation was at 72°C for 5 min. The 16S rRNA PCR product of each (bacterial) isolate was purified and sent for sequencing to LCG genomics (Berlin, Germany). Sequences were identified using NCBI nucleotide database and BLASTn algorithm (Altschul *et al.* 1990). Microscopic pictures to describe the cell morphology were taken and processed with Axio Imager M1 (Carl Zeiss Microscopy GmbH, Germany) in combination with AxioVision V 4.9.1.0 (Carl Zeiss Microscopy GmbH, Germany). All isolates were tested on the resistence against kanamycin and rifampicin by plating them on 0.1 TSB containing 50 µg/ml (final concentration) of each antibiotic. The agar plates were incubated for at least three weeks at 20°C and regularly checked for potential growth.

DNA extraction from fungal hyphae

Hyphae (pre-grown on 0.5 strength PDA) were transferred into Lysin-Matrix E tubes (Biomedicals, The Netherlands), weighted (refer to as fresh-weight), and freeze-dried in liquid nitrogen. Subsequently, fungal-bacterial DNA was extracted with phenol-chloroform

as described by Schulz-Bohm *et al.* (2015). Nucleic acid extracts were stored at -80°C or were immediately used for PCR amplification.

Quantitative PCR of bacterial *rpoB* genes

Primers to quantify the gene of the β subunit of the bacterial RNA polymerase (*rpoB*) were designed based on an alignment of 118 *rpoB* sequences of phylogenetic different bacteria available at NCBI (latest visit: 30/06/15, Table S5.3). The designed primers RpoB-fw1 (5'-GAAGGTCCGAACATCGGTCT-3') and RpoB-r1 (5'-TGCATGTTCGAGCCCATCA-3') amplify a fragment of 370 bp in the conserved region of *rpoB*. Quantitative PCR (qPCR) of the *rpoB* gene was performed with a BioRad C1000 TouchTM Thermal Cycler (Bio-Rad, The Netherlands) whereas each template was quantified in triplicates resulting in a total of nine reactions per fungal isolate (3 technical x 3 biological replicates). The 20-µl reaction mixture consisted of 1-fold SensiFAST™ SYBR® No-ROX Kit (Bioline GmbH, The Netherlands), BSA (0.5 µg µl-1), 500 nM RpoB-fw1, 500 nM RpoB-r1, and 5 µl of diluted template DNA (2-5 ng/µl) or nuclease-free water in case of the negative control. Conditions for the quantification were as followes: 5 min initial denaturation at 95°C, ensued by 40 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 63°C, elongation for 30 s at 72°C, and fluorescence signal detection for 15 s at 82°C. Immediately after the 40th PCR cycle, a melting curve analyses (Figure S5.5) from 63°C to 95°C with increments of 0.5°C was followed. Agarose gel electrophoreses of qPCR products displayed single bands of the expected size. Furthermore, sequencing of cloned *rpoB* qPCR products revealed that solely bacterial *rpoB* genes were targeted by RpoB-fw1/RpoB-r1 despite a huge background of fungal DNA (Table S5.2). Gene copy numbers were calculated according to a standard curve (Figure S5.4) and corrected for potential inhibition (Zaprasis *et al.* 2010). A standard curve was set up by serially diluting of a pGEM-T vector containing an *rpoB* gene fragment of the strain *Burkholderia sp.* AD024.

Cloning of *rpoB* genes and sequence analysis

The *rpoB* qPCR products obtained for DNA extracted from the original fungal isolate M0 or the antibiotic-treated fungal isolate MA were pooled and purified with 20 % polyethylene glycol 6000 (AppliChem, Germany) and 2.5M sodium chloride after following protocol. One volume of PCR product was mixed with one volume of the polyethylene glycol solution, incubated for 15 min at 37°C, and centrifuged for 10 min at 10,000 g. The resulting pellet was washed with 70 % ethanol and resuspended in DNAse/RNAse-free water. Purified qPCR products were ligated into a pGEM-T vector plasmid (Promega, USA) and transformed

in *Escherichia coli* JM109 competent cells (Promega, USA) according to the manual instructions. Clones were picked and checked for the correct insert by M13 PCR (Messing 1993). In total 70 M13 PCR products with the correct length (35 per fungal isolate) were purified and sequenced by LCG genomics (Berlin, Germany).

Sequences were analyzed with MEGA 6 (Tamura *et al.* 2011) and BLASTn. Based on a pairwise distance matrix of aligned *rpoB* nucleotide sequences (Kimura 2-parameter model, substitution includes transitions and transversions, pairwise deletion for gaps/missing data treatment), sequences were assigned to different Operational Taxonomic Units (OTUs) and the diversity index CHAO was calculated with DOTUR (Schloss & Handelsman 2005). A threshold value of 97 % was used to define species-level OTUs (Adékambi *et al.* 2009). The coverage of the gene libraries was calculated according to Schloss *et al.* (2004) and rarefaction curves were constructed after the method of Hurlbert (Hurlbert 1971; Heck *et al.* 1975).

Phylogenetic analyses

All phylogenetic trees were constructed with MEGA 6. The algorithms Maximum-Likelihood (Kimura 2-parameter model, partial deletion, 10,000 bootstraps), Neighbour-Joining (Saitou & Nei 1987; Kimura 2-parameter model, complete deletion, 1000 bootstraps), and Maximum-Parsimony (Subtree-pruning-regrafting search method, complete deletion, 1000 bootstraps) were applied. Trees for 16S rRNA gene sequences from bacterial isolates and *rpoB* clone sequences were based on an alignment of 800 and 398 nucleotide positions, respectively.

Nucleotide sequence accession numbers

Sequences were submitted to GenBank. Accession numbers: KX057404 - KX057471 (*rpoB* clones sequences) and KX057472 - KX057478 (16S rRNA gene sequences of bacterial and yeast isolates associated to M0 and MA).

Volatile trapping and measurement

For the collection of volatiles, glass petri dishes were used with lids with an exit to which a steel trap with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd, United Kingdom) was fixed (Garbeva *et al.* 2014b). Volatiles were collected for 20h at days 3 and 6. The traps were closed and stored at 4°C until analysis. Incubations were done in triplicates, including medium controls.

Trapped volatiles were desorbed using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., United Kingdom) at 210°C for 12 min (Helium flow 50 ml/min) and trapped on a cold trap at -10°C. The trapped volatiles were introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200A QTOF, USA) by heating the cold trap for 3 min to 280°C. Split ratio was set to 1:20, and the column used was a 30 × 0.25 mm ID RXI-5MS, film thickness 0.25 µm (Restek 13424-6850, USA). Temperature program used was as follows: 39°C for 2 min, from 39 to 95°C at 3,5 °C/min, then to 165°C at 6°C/min, to 250°C at 15°C/min and finally to 300°C at 40°C/min, hold 20 min. The VOCs were detected by the MS operating at 70 eV in EI mode. Mass-spectra were extracted with MassHunter Qualitative Analysis Software V B.06.00 Build 6.0.633.0 (Agilent Technologies, USA) using the GC-Q-TOF qualitative analysis module. The obtained mass spectra were exported as mzData files for further processing in MZmine. The files were imported to MZmine V2.14.2 (Pluskal *et al.* 2010) and compounds were identified via their mass spectra using deconvolution function (Local-Maximum algorithm) in combination with two mass-spectral-libraries: NIST 2014 V2.20 (National Institute of Standards and Technology, USA http://www.nist.gov) and Wiley 9th edition mass spectral libraries and by their linear retention indexes (LRI). The LRI values were calculated using an alkane calibration mix before the measurements in combination with AMDIS 2.72 (National Institute of Standards and Technology, USA). The calculated LRI were compared with those found in the NIST and in the in-house NIOO-KNAW LRI database. After deconvolution and mass identification peak lists containing the mass features of each treatment (MZ-value/Retention time and the peak intensity) were created and exported as CSV files for statistical processing via MetaboAnalyst V3.0 (www.metaboanalyst.ca; Xia *et al.* 2015; Xia *et al.* 2012).

Test on the effect of fungal VOCs on bacterial growth and motility

A double plate-within-a-plate system (Schmidt *et al.* 2016) was used to test the effect of fungal volatiles emitted by the M0 and MA isolates on bacterial growth and motility. A 6 mm plug with fungal hyphae (pre-grown on 0.5 strength PDA) was transferred on 1.5 % WA or 0.5 strength PDA (Schmidt *et al.* 2016) on a small Petri-dish (35 mm diameter) located in one half of a bipartite Petri-dish (Figure S5.3). The other half of the bipartite Petri-dish was filled with 12 ml 1.5 % WA + ARE (described above) or with 0.3 % WA + ARE for the motility assay. After the fungus was incubated for three days at 20°C, 10 µl droplets of bacterial suspensions of different cell concentration (10^3-10^6 CFU per ml) were added on WA + ARE, i.e. per replicate four droplets (from lower to higher cell concentration) were placed horizontally to the border of the two compartments (Figure S5.3 A). In case of the motility assay, one droplet of 10 µl bacterial suspension (10^7 CFU per ml) was placed in the middle

of the second compartment filled with 0.3 % motility agar (Figure S4.3 B). The bacterial suspension consisted either of *Burkholderia sp.* AD024 or *Collimonas fungivorans* Ter331 which were grown overnight at 22°C in 0.1 TSB and washed with phosphate buffer (10 mM KH_2PO_4, pH 6.5). Bipartite Petri-dishes were sealed two-times with parafilm and incubated for six days at 20°C.

Colonies at the spot where the lowest dilution of bacterial cells per ml was added were used for the analysis of bacterial growth. In case of bacterial motility, the diameter of the bacterial colony was measured at four different spots (Figure S5.3 B) and a mean value was calculated (refer to as colony extension) for further analyses.

Statistical analysis

Statistical analysis on volatolomic data was performed using MetaboAnalyst V3.0 (Xia *et al.* 2012; Xia *et al.* 2015). To identify significant mass features, One-way-ANOVA with post-hoc Tukey test (HSD- test) were performed. To identify important mass features, PLS-D analysis was performed. Mass features were considered to be statistical relevant if p- and FDR- values were ≤ 0.05. Statistical relevant mass features were further used for compound identification.

The statistical analysis on hyphal extension of *M. hiemalis* as well as on the number of bacterial colonies and on colony extension when they were grown in presence of fungal VOCs was performed with R 3.1.1 (http://www.r-project.org/) using One-way-ANOVA Turkey's HSD test (De Mendiburu 2014). Student's t-tests on data obtained by qPCR as well as for bioassays on fungal growth competition and on fungal growth inhibition by bacterial secondary metabolites were conducted with SigmaPlot 12.5 (Systat Software). The 5% level was taken as threshold for significance.

Results

Composition and abundance of the bacterial community associated with the fungus *M. hiemalis* based on *rpoB* gene

The composition of bacterial community associated with the fungus *M. hiemalies* was determined by targeting the gene of the β subunit of the bacterial RNA polymerase (*rpoB*). For this purpose, primers were newly designed with the aim to amplify a broad spectrum of phylogenetic different bacteria but not fungal DNA. In total 68 *rpoB* clone sequences were retrieved from both the original M0 isolated and antibiotic-treted MA isolate (Figure 5.1 A and Figure S5.2). The total coverage was 94 % and rarefaction analysis showed an out-plateauing curve (Figure 5.1 B) indicating that sequencing was sufficient for the coverage of fungus-associated bacterial species. For M0, the coverage of *rpoB* sequences was lower than for MA (91 % and 97 %, respectively). This is reflected by a stronger out-plateauing rarefaction curve (Figure 5.1 C) and a lower CHAO diversity index for MA compared to M0 (6 and 19, respectively). In general, *rpoB* clone sequences could be assigned to the phyla *Actinobacteria*, *Bacteroidetes*, and *Proteobacteria* (classes: *Alpha-*, *Beta-*, and *Gamma-Proteobacteria*) (Figure S5.2). Clone sequences retrieved from M0 were affiliated to *Actinobacteria* (3 %) and *Proteobacteria* (97 %, dominated by *Alpha-Proteobacteria* [53 %], followed by *Gamma-* and *Beta-Proteobacteria* [29 % and 15 %, respectively]). *RpoB* clone sequences from MA were affiliated with *Bacteroidetes* (3%), and *Proteobacteria* (97 %, dominated by *Gamma-Proteobacteria* [65 %], followed by *Alpha-* and *Beta-Proteobacteria* [24 % and 9 %, respectively]). Within the *rpoB* clone library of M0, most sequences could be assigned to *Bradyrhizobium oligotrophicum* (41 %) and for MA, sequences were mostly related to *Serratia marcescens subsp. sakuensis* (47 %) (Figure 5.1 A).

In total, the bacterial community between M0 and MA appeared to be distinct. More diverse bacteria at the species-level were associated to M0. The bacterial community of the original M0 isolate was dominated by *Alpha-Proteobacteria* (*Bradyrhizobium* and *Brevundimonas*), the community associated to the antibiotic-treated isolate MA appeared to be dominated by *Gamma-Proteobacteria* (*Serratia, Pseudomonas* and *Rhodanobacter*) (Figure 5.1 A). After the antibiotic treatment sequnces of *Mycobacterium* spp., *Stenothrophomonas maltophylia*, *Bradyrhizobium oligotrophicum* and *Bradyrhizobium liaoningense* were not detected.

The PCR quantification revealed that the *rpoB* gene copy number per gram fresh fungal hyphae for M0 ($3.47 \times 10^4 \pm 1.09 \times 10^4$) was about 1.4 fold higher compared to MA ($2.64 \times 10^4 \pm 8.53 \times 10^3$). However, the difference was not significant, indicating that the treatment with broad-spectrum antibiotics reduced the number of associated bacteria to the fungus *M. hiemalis* only slightly.

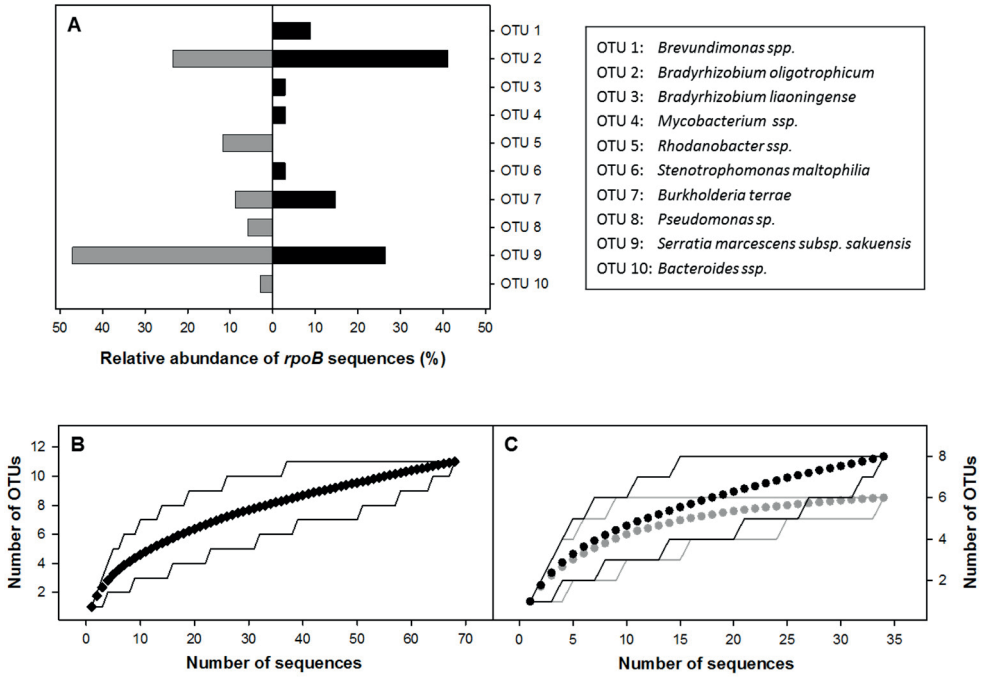
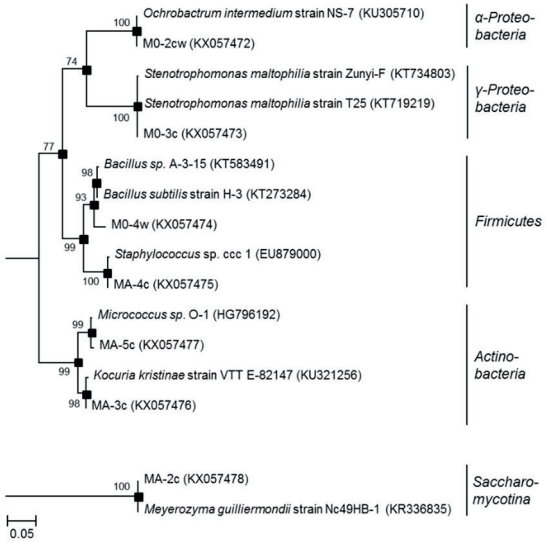


Figure 5.1. Phylogenetic distribution (A) and rarefaction analysis (B and C) of *rpoB* gene sequences from bacteria associated to *M. hiemalis*. Sequences retrieved from *M. hiemalis*, original isolate (M0) and the isolate treated with antibiotics (MA), were assigned to species-level OTUs based on a threshold value of 97% identity. Data corresponding to M0 and MA are indicated in black and grey, respectively. Rarefaction analysis was performed for all sequences obtained from both isolates (B) as well as for the subset per fungal isolate (C). Solid lines in panel B and C represent 95% confidence intervals.

Isolation of *M. hiemalis* associated bacteria and yeast

Fungus-associated bacteria were isolated from both the original M0 and the antibiotic-treated MA isolate by using a combination of three different volatile organic compounds (VOCs) with known antifungal activity (Garbeva *et al.* 2014a). Only by applying a combination of all three volatiles the growth of *Mucor* hyphae could be successfully inhibited while the growth of fungus-associated bacteria was enhanced. In total, seven isolate types with different colony and cell morphologies were obtained (Figure 5.2). Sequences of those isolates were related to different bacteria and to one yeast. Isolates retrieved from M0 were related to the *Alpha-Proteobacterium Ochrobactrum intermedium* strain AG1 (100% identity), the *Gamma-Proteobacterium Stenotrophomonas maltophilia* strain Zunyi-F (99 % identity), and the *Firmicute Bacillus sp.* A-3-15 (100 % identity). Most isolates were related to *S. maltophilia* (Figure 5.3). Sequences related to this species as well as sequences related to

Alpha-Proteobacteria were also found for the *rpoB* clone library of M0 (Figure 5.1 and Figure S5.1). Isolates obtained from MA were related to the *Actinobacterium Kocuria kristinae* strain VTT E-82147 (99 % identity) and *Micrococcus sp.* O-1 (99 % ident), the *Firmicute Staphylococcus sp.* ccc_1 (99 % identity) as well as to the yeast *Meyerozyma guilliermondii* strain Nc49HB-1 (99 % identity). Sequences related to *Staphylococcus* and *Kocuria* were also exclusively detected for MA by next generation sequencing of 16S rRNA (Table S5.2) but not by cloning of *rpoB* qPCR products. All isolates were tested on resistance to rifampicin and kanamycin – antibiotics previously applied to *M. hiemalis*. Only *S. maltophila* and the yeast *Meyerozyma guilliermondii* (obtained from M0 and MA, respectively) grew on TSB with rifampicin and kanamycin (Figure 5.2).



Origin	Isolate type	Colony morphology	Cell morphology	Rif/Amp resistent	Number of isolates related to type
M0	1			No	1
	2			Yes	7
	2			No	3
MA	6			No	1
	4			No	1
	5			No	2
	7			Yes	3

Figure 5.2. Phylogenetic association and characteristics of bacterial and yeast isolates associated to *M. hiemalis*. Bacteria and yeast were isolated from *M. hiemalis,* original isolate (M0) and isolate treated with antibiotics (MA). A Maximum-Likelihood tree of representative 16S rRNA gene sequences was calculated. Percentages of replicate trees (> 50%) in which the associated taxa clustered together in the bootstrap test (10,000 bootstraps) are shown next the branches. Filled squares indicate confirmed tree topology with Maximum-Parsimony and Neighbor Joining (bootstraps > 70 %) calculations with the same dataset. Accession numbers are included in parentheses. Table next to the tree represents general characteristics of the bacterial isolates such as resistance to rifampicin (Rif) and kanamycin (Kan). Scale bar in microcscopic pictures represent 2 µm.

Comparison of the hyphal extension

The comparison of hyphal extension revealed differences between the original M0 isolate and the antibiotic-treated MA isolate on both media, with significantly higher hyphal extension of M0 (Figure 5.3). Besides hyphal extension, morphological differences in pigmentation were observed between the two isolates under both nutrient conditions (FigureS5.4 and 5.5). On the nutrient-rich media, M0 had yellow pigmentation and MA had brown pigmentation (Figure 5.4).

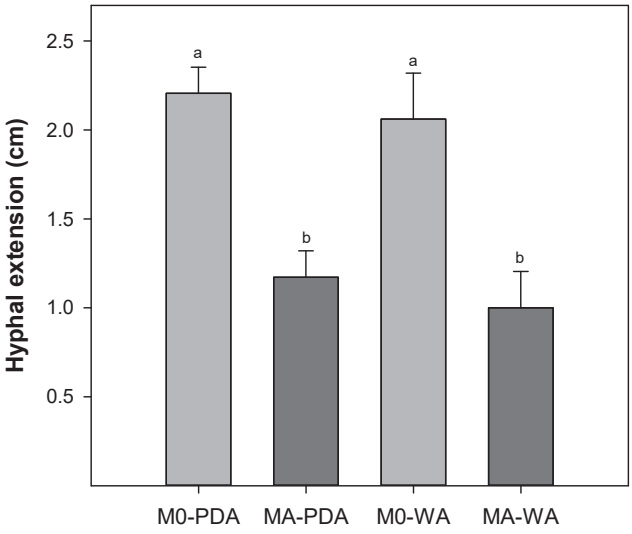


Figure 5.3. Hyphal extension of *M. hiemalis* isolates. Both, the original (M0) and antibiotic-treated isolated (MA) were incubated on 0.5 strength Potato dextrose agar (PDA) or water-agar (WA).

Competition between the fungal isolates M0 and MA

By incubating both fungal isolates M0 and MA together in Petri-dishes, the area of hyphal extension as compared to the monoculture was significantly lower for MA (57 ± 6 % [day7], 35 ± 4 % [day14]; n= 3) than for M0 (74 ± 4 % [day7], 65 ± 3 % [day14]; n = 3). After seven days of incubation, the hyphal extension of both competing isolates did not further increase. Only the hyphal network became denser, and a clear separation zone between both fungal isolates was visible (Figure 5.4). For the control (i.e. the monocultures of both fungal isolates), the Petri-dishes were fully covered with mycelium after 14 days of incubation (Figure 5.4).

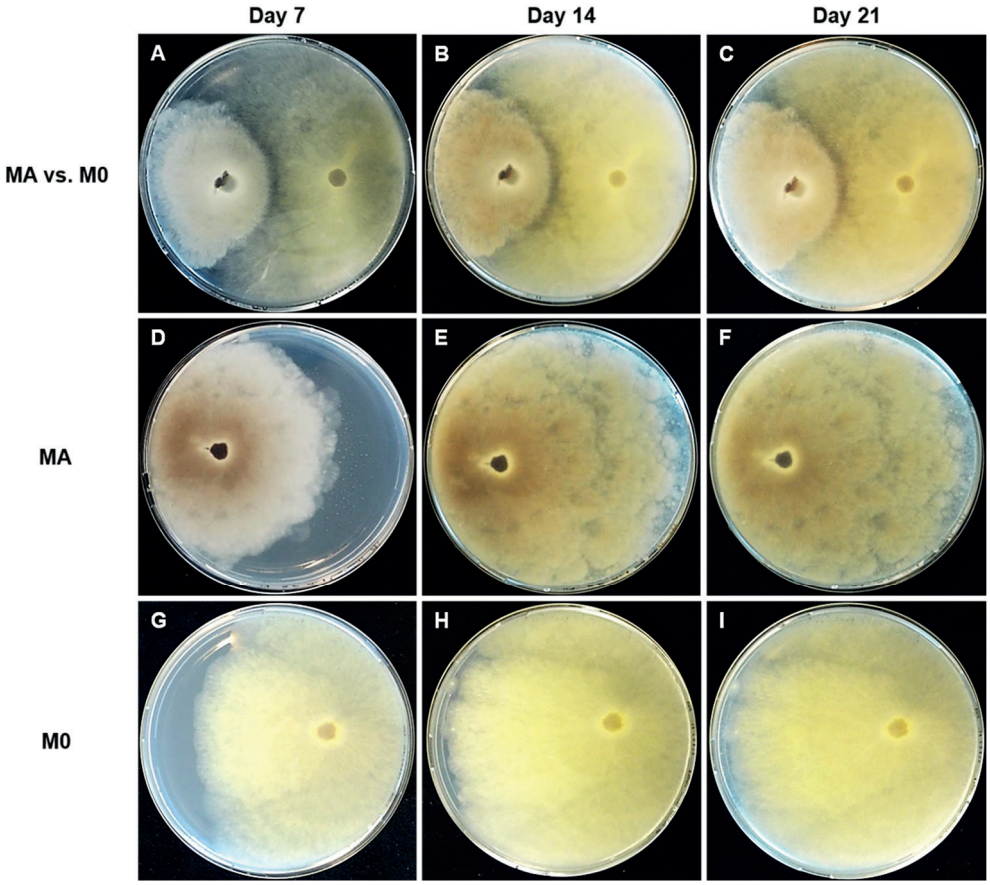


Figure 5.4. Growth of the *M. hiemalis* isolates M0 and MA (original isolate and antibiotic- treated, respectively) on 0.5 strength Potato dextrose agar for 7 (A, D, G), 14 (B, E, H) and 21 days (C, F, I). Fungal isolates were incubated either alone (M0: G-I; MA: D-F) or together (left: MA; right: M0) on one Petri-dish (A-C).

Growth of M0 and MA in presence of rhizobacteria

In the presence of the rhizobacterium *Burkholderia sp*. AD024, the hyphal extension of MA towards the bacterial biofilm was more inhibited as compared to M0 (Figure 5.5 C and I). The inhibition zone, i.e. the distance between bacterial biofilm and the fungal hyphae, was significantly bigger for MA (73 ± 6 mm) than for M0 (43 ± 12 mm).

In case of *Collimonas fungivorans* Ter 331, there was no significant difference between M0 and MA. However, the fungal hyphae of MA were visibly affected by the presence of *Collimonas* compared to M0 (Figure 5.5 B, F, H, and. L). Such difference was not observed for incubations with *Burkholderia sp.* AD024 (Figure 5.5 B, D, H, and J).

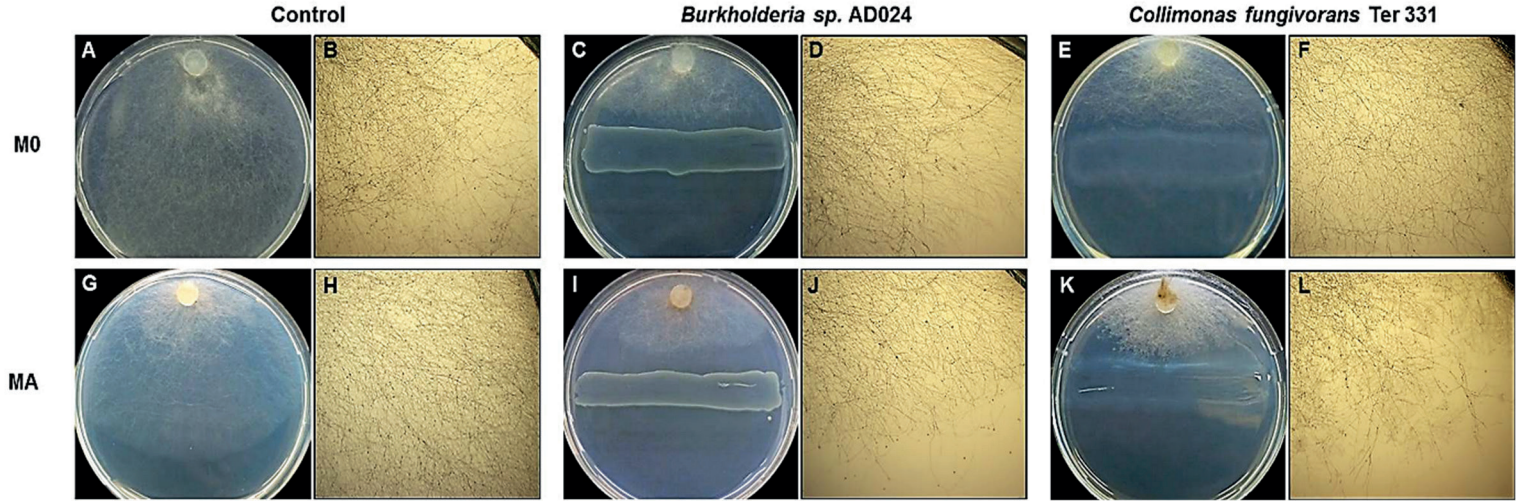


Figure 5.5. Effect of two soil bacteria strains on hyphal extension of the *M. hiemalis* isolates M0 (A-F) and MA (G-L). The bacteria were pre-incubated for three days without fungus followed by a four days incubation with the fungus. M0 represents the original and MA the antibiotic-treated fungal isolate. Overviews of the assays are presented in panels A,C, E, G, I and K and detailed views of mycelia growth in panel B, D, F, H, J and L.

Volatiles produced by *M. hiemalis* isolate M0 and MA

Headspace volatiles produced by *M. hiemalis* M0 and MA on nutrient-rich PDA and nutrient-poor media WA were collected at day 6 after inoculation and subsequently analysed using GC/MS. Different blends of volatiles were produced by the two isolates under both nutrient conditions (Figure 5.6) with more volatile compounds produced on the nutrient-rich PDA media. Interestingly the volatile blend of the MA isolate on nutrient-poor WA media did not differ from the control without fungus (Figure 5.6). Several compounds, such as trifluorbenzene, 1 butanole and 1-butanol-2methyl, were detected only in the headspace of M0 isolate, while heptane-2,4-dimethyl was produced only by the MA isolate (Table 5.1). Numerous compounds could not be assigned with certainty to a volatile organic compound and remained unknown. Some of these unknown compounds were also discriminative between M0 and MA.

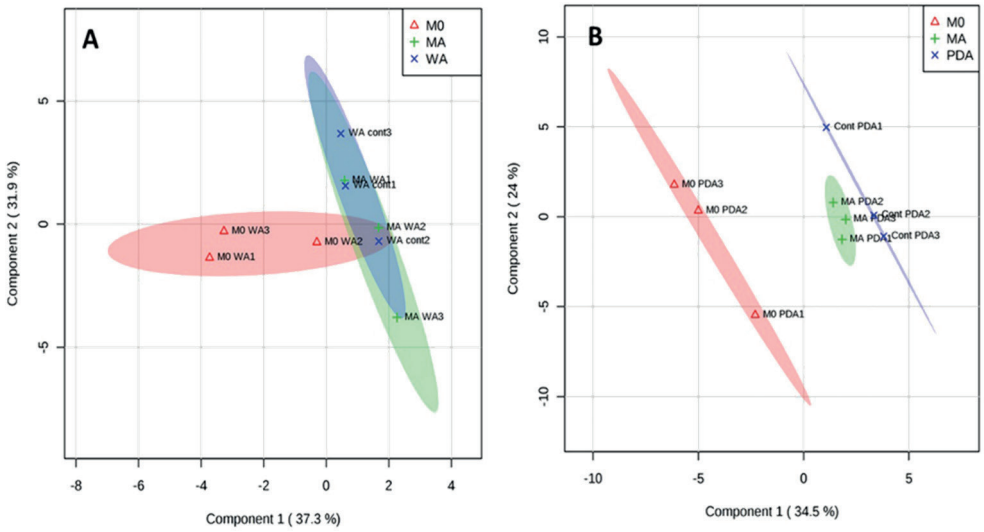


Figure 5.6. PLS-DA score plot of volatile compounds produced by the original (M0) and antibiotic-treated (MA) *M. hiemalis* isolates. A multivariate analysis was performed on mass features of detected compounds which were emitted by the fungi on water-agar (WA; panel A) or potato dextrose agar (PDA; panel B). Controls, volatiles released by the agar media without *M. hiemalis*, are included in the analysis.

Table 5.1. Volatile organic compounds produced by *Mucor hiemalis* on potato dextrose agar.

Compound name	RT	ELRI
3 -Methylpentane	2.8	597
Trifluorbenzene *	3.0	618
1-Butanol*	3.1	620
Carbon tetrachloride*	3.6	657
2-Butanone-3-hydroxy	4.3	708
Unknown *	4.9	730
1-Butanol-2-methyl*	5.0	734
Dimethyl disulfide	5.1	739
Unknown **	6.7	801
Heptane 2,4 dimethyl**	7.2	819
Styrene	9.9	889
Unknown**	13.9	983

*- VOC produced only by *Mucor hiemalis* M0
**- VOC produced only by *Mucor hiemalis* MA
RT - Retention time
ELRI- Experimental linear retention index value

Effect of fungal volatiles on rhizobacteria

By exposure to fungal volatiles emitted by MA on PDA, the numbers of colonies of rhizobacteria *Burkholderia sp*. AD024 and *Collimonas fungivorans* Ter 331 were significantly increased in comparison to M0. Such significant difference was not observed when the fungal isolates were incubated on WA (Figure 5.7 A and B). In addition, the number of *C. fungivorans* Ter 331 colonies was significantly reduced by exposure to volatiles of M0 growing on PDA (Figure 5.7 B).

The motility of both bacteria (reflected in colony extension) was differently affected by the volatiles of M0 and MA (Figure 7C and D). The swimming motility of *C. fungivorans* Ter 331 was significantly higher in presence of volatiles produced by MA growing on PDA as compared to M0 (Figure 5.7 D).

The swimming motility of the bacterial strain *Burkholderia* sp. AD24 was significantly increased by exposure to volatiles of both fungal isolates whereas volatiles of M0 seemed to stimulate more the motility of this strain compared to volatiles of MA (Figure 5.7 C).

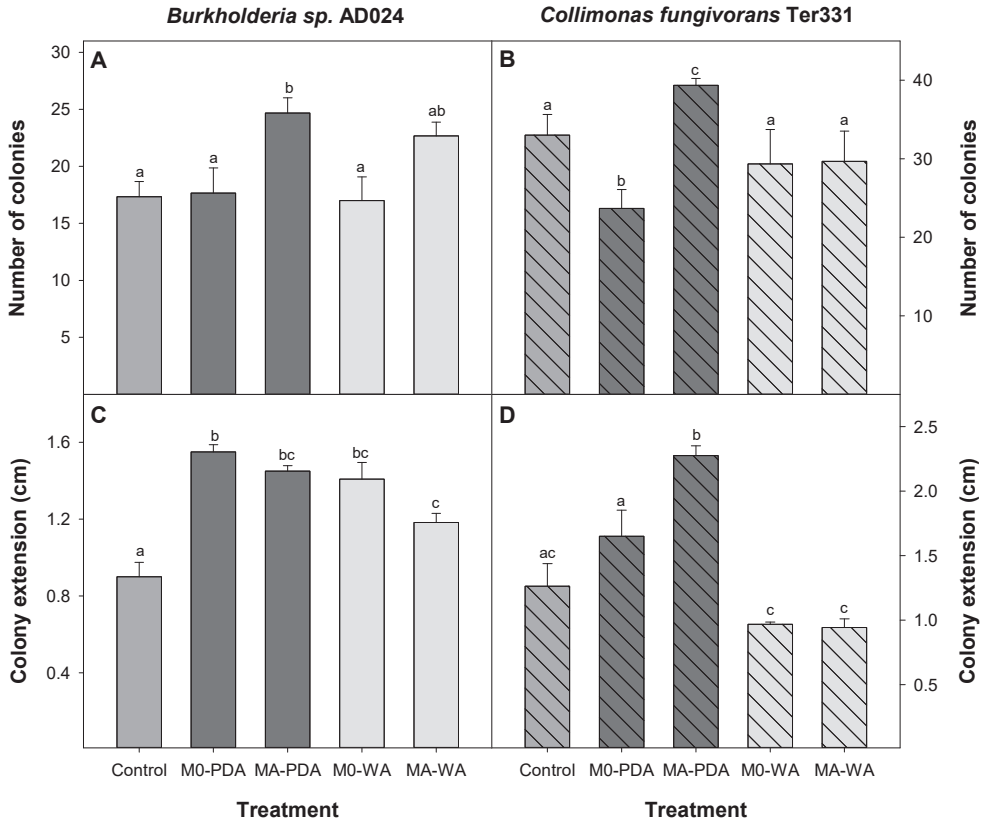


Figure 5.7. Effect of fungal volatiles on the development (A and B) and on swimming motility (C and D) of two rhizobacterial strains. The rhizobacteria were exposed to volatiles produced by original (M0) and antibiotic-treated M. *hiemalis* isolate (MA) growing on 0.5 potato dextrose agar (PDA) or water-agar (WA). Bacterial growth and motility were assessed by counting of colonies appearing at the highest dilution and measuring of the colony extension, respectively. Different letters represent statistical different values tested by One-way-ANOVA.

Discussion

The fates of bacteria and fungi are ecologically intimately connected in soil and rhizosphere. Close association of bacteria with fungi, both endo- and ectosymbionts is well known (Frey-Klett *et al.* 2011). Most methods for fungal isolation and purification are involving application of broad-spectrum antibiotics (Oliveira *et al.* 2013; Singh *et al.* 2015). The model soil-borne fungus *Mucor hiemalis* used in our study was originally isolated by plating on media supplemented with oxytertracycline (De Rooij-van der Goes *et al.* 1995). To ensure that we are using bacteria-free fungus, spores of *Mucor hiemalis* were washed with antibiotics and plated on a media supplemented with rifampicin and kanamycin. The treatment with these broad-spectrum antibiotics strongly altered the fungal morphology and hyphal extension. Hence, the purpose of our study was to determine the bacterial community associated with the fungus *M. hiemalis* and to examine the effect of the two broad-spectrum antibiotics on the fungus-associated bacteriome and, consequently, on fungal behavior and interactions.

Our results revealed that a high diversity of bacteria is associated to *M. hiemalis*. Interestingly, the antibiotic treatment did not significantly reduce the amount of bacteria associated to the fungus but rather changed the community composition by shifting from initially dominating *Alpha-Proteobacteria* to dominance by *Gamma-Proteobacteria*. The antibiotic-treated *M. hiemalis* isolate revealed less diverse bacterial community as compared to the original isolate. Interestingly, several OTUs (*Rhodanobacter* ssp., *Pseudomonas* sp., and *Bacteroides* spp) not detected in the original M0 isolate were detected in the antibiotic treated MA isolates. It is plausible that by affecting some of the antibiotic sensitive strains other "silent" bacteria carried by the fungus became more dominant. The composition of the non-culturable bacterial community associated with the fungus *M. hiemalis* is determined based on housekeeping *rpoB gene* which allowed to target bacteria and, in addition, to avoid co-amplification of fungal DNA. Our attempt to determine the composition of the bacterial community based on 16S rRNA next generation sequencing demonstrated also differences between the bacterial communities (Supplementary material Table S5.2 and Figure S5.1). However, the majority (more than 90%) of OTUs were assigned to fungal mitochondrial DNA (Figure S5.1 A and B).

Plausible explanation for bacterial survival after antibiotic treatment is the resistance to the applied antibiotic or protection by the fungal host. This is in line with another study that failed to obtain bacteria-free fungi (Sharma *et al.* 2008). Analogous, it is also challenging to isolate bacteria that are free from fungal mycelium as often both partners are dependent on each other (Sato *et al.* 2010). Here, we applied a novel method for the isolation of fungal-associated bacteria by the application of three volatile compounds (namely benzonitril,

dimethyldisulfide and dimethyltrisulfide) with strong antifungal activity and stimulating effect on bacterial growth (Garbeva *et al.* 2014a). The high fungal sensitivity to volatile compounds (Effmert *et al.* 2012; Garbeva *et al.* 2014b) compared to high resistance or even growth promotion for bacteria (Garbeva *et al.* 2014a; Schmidt *et al.* 2015) make volatiles suitable compounds for isolation of fungal-associated bacteria. However, difference in fungal susceptibility to volatiles was observed and reported in several independent studies (Effmert *et al.* 2012; Schmidt *et al.* 2015). The yeast *Meyerozyma guilliermondii* was not affected by the volatile treatment indicating that yeast may be less susceptible to volatiles. The results of the bacterial isolation likewise revealed differences in the communities between the original and antibiotic-treated *M. hiemalis*. While isolates obtained from the original M0 isolate belonged to *Alpha-* and *Gamma-Proteobacterium* and *Firmicutes*, the isolates obtained from the antibiotic-treated MA belonged to the phyla *Actinobacteria*, *Firmicutes* and yeast. Interestingly, the test for resistance to antibiotics revealed that only *Stenotrophomonas* and the yeast were resistant to rifampicin and kanamycin, indicating that the fungal host may indeed play a major role in protecting their associated bacteria. Several sequences related to the bacterial isolates such as for *Stenotrophomonas, Staphylococcus* and *Kocuria* were also detected in the *rpoB* clone library and in the next generation 16S rRNA sequencing approach.

The shifts in fungal-associated bacterial community led to clear changes in morphology and behavior of *M. hiemalis*. For instance, the hyphal extension of the original isolate was significantly faster as compared to the antibiotic-treated isolate. The direct competition experiment between the two isolates revealed an advantage in plate colonization for the original M0 isolates indicating that the fast hyphal extension can be beneficial for *M. hiemalis* in terms of resource competition. Whereas bacterial motility in water-unsaturated soil is assumed to be highly restricted, mycelia spread efficiently in the soil, penetrate air-water interfaces and cross over air-filled pores (Jimenez-Sanchez *et al.* 2015; Warmink *et al.* 2011). Hence, fast hyphal extension may be beneficial for bacteria and, therefore, may be stimulated by the *M. hiemalis*-associated bacterial community. Similar stimulation of hyphal growth by bacteria was reported for the interaction of *Amanita muscaria* and *Streptomyces sp*. AcH505 where bacteria showed an enhanced production of the secondary metabolite auxofuran, which promotes the extension of the fungal mycelium (Frey-Klett *et al.* 2011).

Besides hyphal extension, susceptibility to antifungal compounds produced by bacteria was also significantly affected by changes in the *M. hiemalis* associated bacterial community, thereby the antibiotic-treated isolate appeared to be more sensitive to

antifungal compounds as compared to the original isolate. This may point at role of the fungus-associated bacteriome in the protection of its host.

Fungal-associated bacteria have been shown to affect secondary metabolism of the fungi including the production of volatiles (Minerdi *et al.* 2009; Splivallo *et al.* 2015; Vahdatzadeh *et al.* 2015). Due to the shifts in bacterial community, we observed changes in fungal pigmentation and volatile emission. In the last years, it has become evident that microbial volatiles can play major roles in long-distance interactions within soil microbial communities acting as infochemicals or antimicrobial compounds (Effmert *et al.* 2012; Schulz-Bohm *et al.* 2015; Schmidt *et al.* 2015; Schmidt *et al.* 2016). The shifts in fungal-associated bacterial community led to shifts in volatile production of the original and the antibiotic-treated *M. hiemalis* isolates which, consequently, affected their interactions. The volatiles emitted by the original M0 isolate on the nutrient-rich PDA inhibited the growth of *C. fungivorans* Ter 331, whereas the antibiotic-treated MA isolate stimulated the growth of this strain. Bacteria from the genus *Collimonas*, were previously shown to colonize and grow on living fungal hyphae, a phenomenon called mycophagy (De Boer *et al.* 2004; Leveau *et al.* 2010). Interestingly, the motility of *C. fungivorans* Ter 331 was significantly stimulated by the volatiles emitted by the antibiotic-treated *M. hiemalis* implying that volatiles might play a role as long-distance signals for attracting such mycophagous bacteria. Both, volatile-mediated interactions and direct interactions may indicate that the antibiotic-treated isolate is more attractive and susceptible to mycophagous bacterium as compared to the original isolate.

The composition and abundance of volatiles was affected by the nutrient conditions with more volatiles produced on the nutrient-rich PDA media. Several independent studies have reported that the volatile profiles of bacteria and fungi are strongly dependent on growth conditions, interactions and nutrient availability (Garbeva *et al.* 2014b; Schulz-Bohm *et al.* 2015; Tyc *et al.* 2015; Schmidt *et al.* 2016; Weikl *et al.* 2016). Hence, it is questionable whether the same volatile-mediated interactions will occur in nature under nutrient-limited conditions. In soil, the rhizosphere is a "hot-spot" of microbial activity, where approximately 20 to 40% of the phytosynthetic carbon fixed by a plant is released as root exudates (Jones *et al.* 2009; Philippot *et al.* 2013). For a long time, it has been assumed that the rhizosphere is mainly dominated by bacteria, however, recent studies revealed significant utilization of root exudates by saprotrophic fungi (Hannula *et al.* 2012). Therefore, fungal-bacterial interactions as the one described in this study may take place in the rhizosphere and have an effect on the rhizosphere microbiome, which consequently, may play an important role for plant growth and health.

Overall, in the present study we described diverse bacterial community associated with the saprotrophic fungus *M. hiemalis* most probably carried as a contaminant from the natural environment. Our results revealed that antibiotic treatments can cause shifts in this bacterial community that consequently affect the host in terms of morphology, behavior, secondary metabolite production and interactions.

Evidence has emerged over the past years that endo- and ectosymbiotic bacteria are widespread in fungi. Hence, similar to animals and plants, fungi are never alone as they constantly carry their bacteriome. It remains questionable if the fungal isolates in the pure culture collections are really free of bacteria, as the isolation with antibacterials is clearly not sufficient to eradicate the associated bacteria. Future research should be directed to study fungal bacteriome, bacterial localization, the mechanisms of interactions, and its significance for evolution, trophic interactions and ecosystem functioning.

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Supplementary Material

16S rDNA amplification

The composition of the bacterial communities in M0 and MA isolates was determined based on 16S rDNA (V4 region) amplicon sequencing. The 16S rDNA was amplified using the bacterial primer pairs 515F/806R (Caporaso *et al.* 2011) from DNA samples obtained from three replicates per isolate. The PCR reactions were performed with New England BioLabs Phusion High-Fidelity Polymerase (New England BioLabs Cat# M0530L). The PCR reactions were carried out on a Biorad iCycler (Bio-Rad Laboratories B.V., Veenendaal, the Netherlands) and the PCR conditions were as follows: initial cycle 98°C for 30 s. followed by 25 cycles of 98°C for 10 s., 50°C for 30 s. and 72°C for 15 s., followed by a final extension step at 72°C for 7 minutes. Reaction mixtures contained 0.5 µL of forward and reverse primer (5 µM of each primer), 10 µL of 5 x New England BioLabs Phusion High-Fidelity Polymerase reaction buffer, 1 µl of 10 mM DNTPs, 1.5 µl DMSO, 3 µl of template DNA, 0.5 µl Phusion High-Fidelity polymerase and 33 µl nuclease free water resulting in a final reaction volume of 50 µL. After PCR amplification all samples were put on a 1.0 % agarose gel to check the size and integrity of the amplified DNA. The PCR products of the antibiotic-treated *Mucor hiemalis*. (MA) were cut out of the agarose gel and purified with the Qiagen gel extraction kit (Qiagen Cat# 28706). The PCR products of all samples were purified with the AMPure XP PCR Purification Kit (Agencourt Cat# A638881) and quantified with Qubit Fluorometer (Thermo Fisher, Invitrogen). The samples were then combined in equimolar ratios and subjected to IonTorrent™ sequencing.

IonTorrent sequencing and 16S rDNA data analysis

The DNA fragments were sequenced using IonTorrent™ semiconductor technology (Thermo Fisher Scientific) for unidirectional sequencing of the amplicon libraries. Barcoded primers were used to multiplex the amplicon pools in order to be able to sequence the samples together and to computationally separate the sample afterwards. The barcode (Table S5.1) of 12 bases was added to the reverse primer 806R and unidirectional sequencing was performed from the A-key adapter. A two-base linker sequence was inserted between the adapter and the 16S rDNA primers to reduce any effect of the composite primer on PCR efficiency. Ion OneTouch™ 2 System and Ion PGM™ Template OT2 400 Kit Template were used for library preparation and the sequencing was performed using HiQ Ion PGM™ Sequencing 400 on Ion PGM™ System using Ion 318™ Chip v2. From the obtained sequence data an OTU-table was built as follows: Forward and reverse primer sequences were removed from the FASTQ files using Flexbar version 2.5 (Dodt *et al.* 2012). The sequences

were trimmed based on sequence quality with a minimum quality of 25 and a minimum length of 150 bp. Sequences were converted to FASTA format and concatenated into a single file. All reads were clustered into OTUs using the UPARSE strategy by de-replication, sorted by abundance with at least two sequences and clustered using the UCLUST smallmem algorithm (Edgar 2010). These steps were performed with VSEARCH version 1.0.10 (Rognes *et al.* 2016). Next, the obtained sequences were checked for chimeric sequences using the UCHIME algorithm (Edgar *et al.* 2011) implemented in VSEARCH. All reads were mapped to OTUs before the de-replication using the usearch_global method of VSEARCH to create an OTU-table and converted to BIOM-Format 1.3.1 (McDonald *et al.* 2012). Finally, taxonomic information for each OTU was added to the BIOM file by using the RDP Classifier version 2.10 (Cole *et al.* 2014).

Table S5.1. List of primers used in this study amplifying the V4 region of the 16S rDNA. Barcodes of the primers are in bold red letters, the linker sequence is shown in blue letters.

Primers used for 16S rDNA sequencing		
Primer code	Sequence (5' - 3') of primers barcode in **red bold letters** / two base linker in blue	**Barcode sequence**
515F – P1	5'**CCTCTCTATGGGCA**GTCGGTGATGTGTGCCAGCMGCCGCGGTAA	CCTCTCTATGGGCA
806R – E60	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**CGGTCAATTGAC**CCGGACTACHVGGGTWTCTAAT	CGGTCAATTGAC
806R – E61	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**GTGGAGTCTCAT**CCGGACTACHVGGGTWTCTAAT	GTGGAGTCTCAT
806R – E77	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**AGCATGTCCCGT**CCGGACTACHVGGGTWTCTAAT	AGCATGTCCCGT
806R – E78	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**GTACGATATGAC**CCGGACTACHVGGGTWTCTAAT	GTACGATATGAC
806R – E79	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**GTGGTGGTTTCC**CCGGACTACHVGGGTWTCTAAT	GTGGTGGTTTCC
806R – E80	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**TAGTATGCGCAA**CCGGACTACHVGGGTWTCTAAT	TAGTATGCGCAA
806R – E81	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**TGCGCTGAATGT**CCGGACTACHVGGGTWTCTAAT	TGCGCTGAATGT
806R – E105	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**ACTTGGTGTAAG**CCGGACTACHVGGGTWTCTAAT	ACTTGGTGTAAG
806R – E106	5'CCATCTCATCCCTGCGTGTCTCCGACTCAG**TCTTGGAGGTCA**CCGGACTACHVGGGTWTCTAAT	TCTTGGAGGTCA

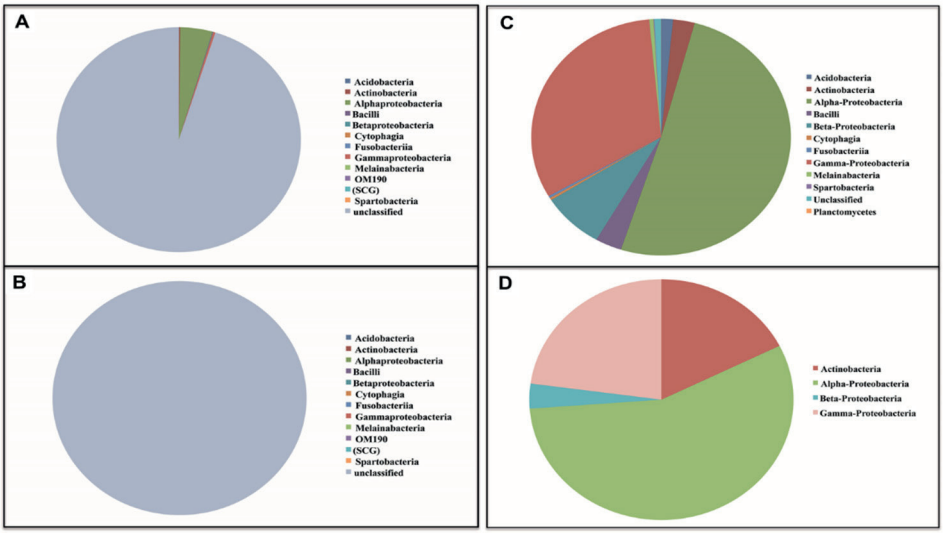

Figure S5.1. Pie charts of the relative abundance of 16S rDNA clusters retrieved from fungus-associated bacteria of the antibiotic-treated (A and C) and original (B and D) *M. hiemalis* isolate as well as fungal mitochondria (unclassified). Graph C and D represents data of bacterial 16S rDNA clusters only.

Table S5.2. Phylogenetic distribution of bacterial 16S rDNA associated to *M. hiemalis*. Sequences were retrieved from non-treated (M0) and antibiotic-treated *M. hiemalis* isolates (MA).

OTU	Number of Sequences						Taxonomy	Closest BLAST hit	E-value	Identity
	M0-1	M0-2	M0-3	MA-1	MA-2	MA-3				
12	2	0	4	2	32	23222	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured *Paracoccus* sp. partial 16S rRNA gene, DGGE band B178	1.00E-128	uncultured *Paracoccus* sp.
14	1	2	0	3	32	6274	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured bacterium gene for 16S ribosomal RNA, partial sequence, clone: Exp331_INH_67U_48	1.00E-128	uncultured bacterium
25	0	0	0	0	9	2576	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured *Paracoccus* sp. clone H1 16S ribosomal RNA gene, partial sequence	3.00E-124	uncultured *Paracoccus* sp.
49	0	0	0	0	2	2040	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured bacterium clone ncd119c03c1 16S ribosomal RNA gene, partial sequence	3.00E-120	uncultured bacterium
35	0	1	0	0	4	1952	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured *Paracoccus* sp. clone H1 16S ribosomal RNA gene, partial sequence	3.00E-124	uncultured *Paracoccus* sp.
42	0	0	0	0	2	1893	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured bacterium partial 16S rRNA gene, clone U18	9.00E-125	uncultured bacterium
33	0	1	1	0	4	1836	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured bacterium clone ncd1105c05c1 16S ribosomal RNA gene, partial sequence	2.00E-121	uncultured bacterium
36	0	2	1	3	650	679	*Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae; Pseudomonas;*	*Pseudomonas mandelii* strain YSP-Y1 16S ribosomal RNA gene, partial sequence	1.00E-128	*Pseudomonas mandelii*

Table S5.2 continued.

OTU	Number of Sequences						Taxonomy	Closest BLAST hit	E-value	Identity
	M0-1	M0-2	M0-3	MA-1	MA-2	MA-3				
91	0	0	0	0	2	787	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured bacterium clone COB27 16S ribosomal RNA gene, partial sequence	6.00E-112	uncultured bacterium
68	0	0	0	0	0	685	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured bacterium clone ncd128g12c1 16S ribosomal RNA gene, partial sequence	6.00E-122	uncultured bacterium
55	0	0	0	0	0	620	*Bacteria; Actinobacteria; Actinobacteria; Micrococcales; Micrococcaceae; Kocuria;*	*Kocuria* sp. PN8 16S ribosomal RNA gene, partial sequence	1.00E-128	*Kocuria* sp. PN8
60	0	2	0	1	1	512	*Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Moraxella;*	Uncultured bacterium clone MBR640 16S ribosomal RNA gene, partial sequence	3.00E-129	uncultured bacterium
57	1	0	0	6	259	209	*Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia-Shigella;*	Chain A, Visualization Of Two Trnas Trapped In Transit During Ef-g-mediated Translocation (30s Subunit + Ligands)	1.00E-128	*Escherichia coli* K-12
59	0	1	0	0	6	411	*Bacteria; Actinobacteria; Actinobacteria; Micrococcales; Micrococcaceae; Micrococcus;*	*Micrococcaceae* bacterium 6_Ne_3 16S ribosomal RNA gene, partial sequence	1.00E-128	*Micrococcaceae* bacterium 6_Ne_3
75	0	1	0	1	96	230	*Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae;*	*Variovorax* sp. MG3 16S ribosomal RNA gene, partial sequence	1.00E-128	*Variovorax* sp. MG3
63	0	0	0	0	13	303	*Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter;*	Uncultured bacterium partial 16S rRNA gene, clone LE21_39	1.00E-128	uncultured bacterium
61	0	0	0	0	52	250	*Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Sphingomonas;*	*Sphingomonas sp.* 1036 16S ribosomal RNA gene, partial sequence	1.00E-128	*Sphingomonas sp.* 1036

Table S5.2 continued.

OTU	Number of Sequences						Taxonomy	Closest BLAST hit	E-value	Identity
	M0-1	M0-2	M0-3	MA-1	MA-2	MA-3				
69	0	0	0	0	0	262	*Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus;*	Uncultured bacterium isolate DGGE gel band 03_D5 clone 01 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
70	0	0	1	0	144	82	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; Methylobacterium;*	Uncultured bacterium isolate DGGE gel band A066 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
67	0	0	0	0	61	116	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Bradyrhizobiaceae; Bradyrhizobium;*	*Bradyrhizobium sp.* 22 16S ribosomal RNA gene, partial sequence	1.00E-128	*Bradyrhizobium sp.* 22
76	0	0	0	0	1	151	*Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae;*	Uncultured bacterium clone lp038 16S ribosomal RNA gene, partial sequence	6.00E-127	uncultured bacterium
72	0	0	0	0	0	149	*Bacteria; Acidobacteria; Acidobacteria; Subgroup_6;*	Uncultured bacterium isolate DGGE gel band 02_P1 clone 06 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
80	0	0	0	1	95	48	*Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae; Staphylococcus;*	Staphylococcus hominis gene for 16S ribosomal RNA, partial sequence, strain: DAT706	1.00E-128	*Staphylococcus hominis*
87	1	0	0	0	0	140	*Bacteria; Actinobacteria; Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacterium_1;*	*Corynebacterium sp.* canine oral taxon 424 strain OH977 16S ribosomal RNA gene, partial sequence	3.00E-129	*Corynebacterium sp.* canine oral taxon 424
98	0	0	0	1	0	123	*Bacteria; Actinobacteria; Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacterium_1;*	*Corynebacterium* sp. 19.L-CHEST 16S ribosomal RNA gene, partial sequence	3.00E-129	*Corynebacterium sp.* 19.L-CHEST

Table S5.2 continued.

OTU	Number of Sequences						Taxonomy	Closest BLAST hit	E-value	Identity
	M0-1	M0-2	M0-3	MA-1	MA-2	MA-3				
81	0	0	0	0	0	107	*Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Tepidimonas;*	Uncultured bacterium clone AB94 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
71	0	0	0	0	40	61	*Bacteria; Firmicutes;*	*Streptococcus* thermophilus strain CGLBL208 16S ribosomal RNA gene, partial sequence	4.00E-97	*Streptococcus thermophilus*
90	0	0	0	0	8	87	*Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Caulobacter;*	Uncultured bacterium clone S18-3 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
89	0	0	0	2	78	13	*Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Burkholderia;*	*Burkholderia* pseudomallei strain DRDEBPS1017 16S ribosomal RNA gene, partial sequence	1.00E-128	*Burkholderia pseudomallei*
86	0	0	0	0	0	87	*Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae; Ralstonia;*	Uncultured bacterium clone W3-76 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
97	0	0	0	0	0	83	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; Methylobacterium;*	Uncultured bacterium clone GXTJ5A301BHXSL 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
94	0	0	0	1	0	70	*Bacteria; Actinobacteria; Actinobacteria; Micrococcales; Dermabacteraceae; Brachybacterium;*	Uncultured bacterium clone S22_055 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
82	0	0	0	1	0	0	*Bacteria; Acidobacteria; Acidobacteria; Subgroup_6;*	Uncultured bacterium clone FFCH11863 16S ribosomal RNA gene, partial sequence	3.00E-129	uncultured bacterium
99	0	0	0	0	0	69	Unclassified;	Uncultured bacterium isolate DGGE gel band 02_M2 clone 03 16S ribosomal RNA gene, partial sequence	3.00E-100	uncultured bacterium

Table S5.2 continued.

OTU	Number of Sequences						Taxonomy	Closest BLAST hit	E-value	Identity
	M0-1	M0-2	M0-3	MA-1	MA-2	MA-3				
113	0	0	0	0	0	64	*Bacteria; Actinobacteria; Actinobacteria; Micrococcales; Brevibacteriaceae; Brevibacterium;*	*Brevibacterium* luteolum strain DNF00447 16S ribosomal RNA gene, partial sequence	1.00E-128	*Brevibacterium luteolum*
122	0	0	0	0	0	61	*Bacteria; Actinobacteria; Actinobacteria; Frankiales; Geodermatophilaceae; Blastococcus;*	Uncultured *Geodermatophilus* sp. clone BC079 16S ribosomal RNA gene, partial sequence	6.00E-127	uncultured *Geodermato-philus sp*.
84	0	0	0	0	45	14	*Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas;*	*Brevundimonas* sp. 8_65 partial 16S rRNA gene, strain 8_65	3.00E-114	*Brevundimonas sp*. 8_65
114	0	0	0	0	0	58	*Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; Bacillus;*	Uncultured bacterium isolate DGGE gel band 03_D5 clone 01 16S ribosomal RNA gene, partial sequence	3.00E-119	uncultured bacterium
120	2	0	0	0	0	54	*Bacteria; Actinobacteria; Actinobacteria; Corynebacteriales; Corynebacteriaceae;*	Uncultured bacterium clone RTKG4A-A06 16S ribosomal RNA gene, partial sequence	3.00E-129	uncultured bacterium
131	0	0	0	0	0	56	*Bacteria; Verrucomicrobia; Spartobacteria; Chthoniobacterales; Xiphinematobacteraceae; Candidatus_Xiphinematobacter;*	Uncultured bacterium clone p8f04ok 16S ribosomal RNA gene, partial sequence	6.00E-127	uncultured bacterium
100	0	0	0	0	0	53	*Bacteria; Verrucomicrobia; Spartobacteria; Chthoniobacterales; Chthoniobacteraceae; Chthoniobacter;*	Uncultured bacterium clone 4-9 16S ribosomal RNA gene, partial sequence	7.00E-126	uncultured bacterium
116	0	0	0	0	0	52	Unclassified;	Uncultured bacterium clone MNII13C29-B91 small subunit ribosomal RNA gene, partial sequence	3.00E-125	uncultured bacterium

Table S5.2 continued.

OTU	Number of Sequences						Taxonomy	Closest BLAST hit	E-value	Identity
	M0-1	M0-2	M0-3	MA-1	MA-2	MA-3				
119	0	0	0	0	27	23	*Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae; Acinetobacter;*	Uncultured bacterium isolate DGGE gel band A105 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
108	0	0	0	0	41	1	*Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Brevundimonas;*	Uncultured bacterium clone B346 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
136	0	0	0	0	4	33	*Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Stenotrophomonas;*	*Stenotrophomonas* maltophilia strain S2 16S ribosomal RNA gene, partial sequence	1.00E-128	*Stenotrophomonas maltophilia*
106	0	0	0	0	31	0	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; Methylobacterium;*	Uncultured bacterium isolate DGGE gel band A064 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
109	0	0	0	0	31	0	*Bacteria; Cyanobacteria; Melainabacteria; Obscuribacterales;*	Uncultured bacterium RNA for 16S rRNA, partial sequence, clone: B1001R003_G22	3.00E-125	uncultured bacterium
110	0	0	0	0	16	12	*Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Escherichia-Shigella;*	Uncultured bacterium clone S2C222 16S ribosomal RNA gene, partial sequence	1.00E-108	uncultured bacterium
115	0	0	0	0	0	28	*Bacteria; Actinobacteria; Actinobacteria; Propionibacteriales; Nocardioidaceae;*	Uncultured bacterium clone A3-303. 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
128	0	0	0	0	27	0	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Hyphomicrobiaceae; Pedomicrobium;*	Uncultured alpha proteobacterium partial 16S rRNA gene, clone F90B0W9	1.00E-128	uncultured alphaproteo bacterium

Table S5.2 continued.

OTU	Number of Sequences						Taxonomy	Closest BLAST hit	E-value	Identity
	M0-1	M0-2	M0-3	MA-1	MA-2	MA-3				
128	0	0	0	0	0	13	*Archaea; Thaumarchaeota; Soil_Crenarchaeotic_Group(SCG);*	Uncultured archaeon clone QRS_17 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured archaeon
112	0	0	0	0	0	26	*Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Streptococcus;*	Uncultured bacterium clone KSD2_19 16S ribosomal RNA gene, partial sequence	4.00E-97	uncultured bacterium
111	0	0	0	0	22	0	*Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Comamonas;*	Uncultured bacterium clone Mackrl2_A11 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured bacterium
141	0	0	0	0	0	20	*Bacteria; Actinobacteria; Actinobacteria; Micrococcales; Micrococcaceae; Kocuria;*	Uncultured bacterium clone ncd1273e05c1 16S ribosomal RNA gene, partial sequence	3.00E-120	uncultured bacterium
107	0	0	0	0	18	0	*Bacteria; Bacteroidetes; Cytophagia; Cytophagales; Cytophagaceae; Hymenobacter;*	Uncultured bacterium partial 16S rRNA gene, clone 3_H10	1.00E-128	uncultured bacterium
118	0	0	0	0	17	0	*Bacteria; Fusobacteria; Fusobacteriia; Fusobacteriales; Fusobacteriaceae; Fusobacterium;*	*Fusobacterium nucleatum* strain YWH7071 16S ribosomal RNA gene, partial sequence	7.00E-90	*Fusobacterium nucleatum*
105	0	0	0	0	4	11	*Bacteria; Actinobacteria; Actinobacteria; Corynebacteriales; Corynebacteriaceae; Corynebacterium_1;*	Bacterium 114 16S ribosomal RNA gene, partial sequence	3.00E-129	bacterium 114
139	0	0	0	0	0	15	*Bacteria; Actinobacteria; Actinobacteria; Micrococcales; Intrasporangiaceae;*	*Janibacter limosus* partial 16S rRNA gene, strain B0210-9I4	1.00E-128	Janibacter limosus
133	0	0	0	0	0	14	*Bacteria; Planctomycetes; OM190;*	Uncultured bacterium clone Paddy_500_9202 16S ribosomal RNA gene, partial sequence	1.00E-123	uncultured bacterium

Table S5.2 continued.

OTU	Number of Sequences						Taxonomy	Closest BLAST hit	E-value	Identity
	M0-1	M0-2	M0-3	MA-1	MA-2	MA-3				
135	0	0	0	0	0	13	*Archaea; Thaumarchaeota; Soil_Crenarchaeotic_Group(SCG);*	Uncultured archaeon clone QRS_17 16S ribosomal RNA gene, partial sequence	1.00E-128	uncultured archaeon
126	0	0	0	3	9	0	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Phyllobacteriaceae; Mesorhizobium;*	*Chelativorans sp.* B0.09-98 16S ribosomal RNA gene, partial sequence	1.00E-128	*Chelativorans sp.* B0.09-98
137	0	0	0	0	0	12	*Bacteria; Firmicutes; Bacilli; Lactobacillales; Streptococcaceae; Lactococcus;*	*Lactococcus lactis* strain A8 16S ribosomal RNA gene, partial sequence	4.00E-128	*Lactococcus lactis*
121	0	0	0	0	0	11	*Bacteria; Actinobacteria; Actinobacteria; Frankiales; Geodermatophilaceae; Modestobacter;*	Modestobacter versicolor strain ZSGR36 16S ribosomal RNA gene, partial sequence	1.00E-128	*Modesto bacter versicolor*
132	0	0	0	1	2	7	*Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Caulobacteraceae; Caulobacter;*	Uncultured bacterium clone PM_S1_30 16S ribosomal RNA gene, partial sequence	4.00E-118	uncultured bacterium
138	0	0	0	0	0	5	*Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Paracocccus;*	Uncultured *Paracoccus sp.* partial 16S rRNA gene, DGGE band B178	6.00E-117	uncultured *Paracoccus sp.*
127	0	0	0	0	4	0	*Bacteria; Proteobacteria; Alphaproteobacteria; Sphingomonadales; Sphingomonadaceae; Novosphingobium;*	Uncultured bacterium partial 16S rRNA gene, clone LE21_28	1.00E-128	uncultured bacterium
130	0	0	0	0	0	3	*Bacteria; Actinobacteria; Actinobacteria; Corynebacteriales; Nocardiaceae; Gordonia;*	*Gordonia paraffinivorans* strain A4-16 16S ribosomal RNA gene, partial sequence	1.00E-128	*Gordonia paraffinivorans*
140	0	0	0	0	0	2	Unclassified;	16S rRNA amplicon fragment from a soil sample (ferralsol, Madagascar) resulting from a 16 days laboratory incubation experiment in the presence of 13C-enriched wheat-straw : Light-DNA fraction (DNA-SIP technique)	3.00E-73	unidentified

Table S5.3. Available *rpoB* gene sequences of bacteria retrieved from NCBI (latest visit: 30/06/15) which were used to design *rpoB* primers.

Accession number	Bacterial strain	Family	Phyla
AY242821	*Afipia birgiae* strain 34632	*Bradyrhizobiaceae*	*(Alpha-) Proteobacteria*
AY242822	*Afipia broomeae* strain B-91-007286	*Bradyrhizobiaceae*	*(Alpha-) Proteobacteria*
AY242823	*Afipia clevelandensis* strain B-91-007353	*Bradyrhizobiaceae*	*(Alpha-) Proteobacteria*
AF237414	*Anaplasma phagocytophilum*	*Anaplasmataceae*	*(Alpha-) Proteobacteria*
AF171070	*Bartonella henselae* Houston-1	*Bartonellaceae*	*(Alpha-) Proteobacteria*
AF165994	*Bartonella quintana*	*Bartonellaceae*	*(Alpha-) Proteobacteria*
AY242836	*Bosea massiliensis* strain 34639	*Bradyrhizobiaceae*	*(Alpha-) Proteobacteria*
AY242833	*Bosea minatitlanensis* strain AMX51	*Bradyrhizobiaceae*	*(Alpha-) Proteobacteria*
AY242832	*Bosea thiooxidans* strain BI-42	*Bradyrhizobiaceae*	*(Alpha-) Proteobacteria*
AY242830	*Bradyrhizobium japonicum* strain 3I1b6	*Bradyrhizobiaceae*	*(Alpha-) Proteobacteria*
AY242831	*Bradyrhizobium liaoningense* strain ESG2281	*Bradyrhizobiaceae*	*(Alpha-) Proteobacteria*
AY562181	*Brucella melitensis biovar Abortus* clone Ba3R	*Brucellaceae*	*(Alpha-) Proteobacteria*
KP720639	*Candidatus Bartonella ancashi* isolate 20	*Bartonellaceae*	*(Alpha-) Proteobacteria*
HM072453	*Candidatus Odyssella thessalonicensis* clone 1111798	*Candidatus Paracaedibacteraceae*	*(Alpha-) Proteobacteria*
DQ186620	*Micavibrio sp.* EPB	*unclassified Alphaproteobacteria*	*(Alpha-) Proteobacteria*
AF401089	*Neorickettsia risticii*	*Anaplasmataceae*	*(Alpha-) Proteobacteria*
JQ757054	*Rhizobium etli* strain Mim1	*Rhizobiaceae*	*(Alpha-) Proteobacteria*
KF836417	*Rhizobium grahamii* strain CCGM3	*Rhizobiaceae*	*(Alpha-) Proteobacteria*
KF836415	*Rhizobium leguminosarum bv. phaseoli* strain CCGM8	*Rhizobiaceae*	*(Alpha-) Proteobacteria*
AF034531	*Rickettsia prowazekii*	*Rickettsiaceae*	*(Alpha-) Proteobacteria*
AF236795	*Rickettsia pulicis*	*Rickettsiaceae*	*(Alpha-) Proteobacteria*
AF401090	*Wolbachia pipientis*	*Anaplasmataceae*	*(Alpha-) Proteobacteria*
DQ420116	*Achromobacter xylosoxidans* strain ATCC 15173 AH015723SEG	*Alcaligenaceae*	*(Beta-) Proteobacteria*
FJ214311	*Verminephrobacter sp.* At4	*Comamonadaceae*	*(Beta-) Proteobacteria*
CP002031	*Geobacter sulfurreducens* KN400	*Geobacteraceae*	*(Delta-) Proteobacteria*
KC408834	*Campylobacter jejuni subsp. jejuni* strain H704a	*Campylobacteraceae*	*(Epsilon-) Proteobacteria*
FJ715473	*Acinetobacter baumannii strain* Ab03-128	*Moraxellaceae*	*(Gamma-) Proteobacteria*
DQ207486	*Acinetobacter junii* strain CIP 64.5	*Moraxellaceae*	*(Gamma-) Proteobacteria*
DQ207487	*Acinetobacter lwoffii* strain CIP 64.1	*Moraxellaceae*	*(Gamma-) Proteobacteria*
KJ919956	*Actinobacillus pleuropneumoniae strain APBUAP*	*Pasteurellaceae*	*(Gamma-) Proteobacteria*

Table S5.3 continued.

Accession number	Bacterial strain	Family	Phyla
EU684590	*Actinosynnema pretiosum* subsp. auranticum strain 31565	*Pasteurellaceae*	*(Gamma-) Proteobacteria*
KC133527	*Aeromonas hydrophila* strain AH11Novo	*Aeromonadaceae*	*(Gamma-) Proteobacteria*
KP177958	*Coxiellaceae* bacterium CC99	*Coxiellaceae*	*(Gamma-) Proteobacteria*
KC665638	*Edwardsiella tarda* strain Eta30305Parent	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
AY167138	*Enterococcus faecium* isolate 343-3	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
KP670778	*Escherichia coli* strain DA14704	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
JN707608	*Escherichia coli* strain E10	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
JF290381	*Francisella halioticida* strain Shimane-1	*Francisellaceae*	*(Gamma-) Proteobacteria*
EU683010	*Francisella noatunensis* subsp. orientalis strain	*Francisellaceae*	*(Gamma-) Proteobacteria*
EU723255	*Francisella philomiragia* subsp. noatunensis strain 2005/50/F292-6C	*Francisellaceae*	*(Gamma-) Proteobacteria*
JQ894910	*Gilliamella apicola* strain wkB1	*Orbaceae*	*(Gamma-) Proteobacteria*
KC253224	*Haliea rubra* DSM 19751	*Halieaceae*	*(Gamma-) Proteobacteria*
JN377745	*Klebsiella pneumoniae* subsp. pneumoniae strain Kp13	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
KM458063	*Lelliottia sp.* GL2	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
HQ729479	*Pseudoalteromonas sp*. D41	*Pseudoaltero-monadaceae*	*(Gamma-) Proteobacteria*
KF206426	*Pseudomonas fluorescens* strain EXXP-01	*Pseudomonadaceae*	*(Gamma-) Proteobacteria*
JQ728859	*Salmonella enterica* subsp. enterica serovar Agona strain B15	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
KM350528	*Thorsellia anophelis*	*Thorselliaceae*	*(Gamma-) Proteobacteria*
KM350529	Thorsellia sp. T2.1	*Thorselliaceae*	*(Gamma-) Proteobacteria*
KM350530	*Thorsellia sp*. W5.1.1	*Thorselliaceae*	*(Gamma-) Proteobacteria*
HM622073	*Vibrio vulnificus* strain 1.1758	*Vibrionaceae*	*(Gamma-) Proteobacteria*
HQ729479	*Pseudoalteromonas sp.* D41	*Pseudoaltero-monadaceae*	*(Gamma-) Proteobacteria*
KF206426	*Pseudomonas fluorescens* strain EXXP-01	*Pseudomonadaceae*	*(Gamma-) Proteobacteria*
JQ728859	*Salmonella enterica subsp. enterica* serovar Agona strain B15	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
KM350528	*Thorsellia anophelis*	*Thorselliaceae*	*(Gamma-) Proteobacteria*
KM350529	*Thorsellia sp.* T2.1	*Thorselliaceae*	*(Gamma-) Proteobacteria*
KM350530	*Thorsellia sp*. W5.1.1	*Thorselliaceae*	*(Gamma-) Proteobacteria*
HM622073	*Vibrio vulnificus* strain 1.1758	*Vibrionaceae*	*(Gamma-) Proteobacteria*

Table S5.3 continued.

Accession number	Bacterial strain	Family	Phyla
AF426390	*Xanthomonas campestris pv. campestris*	*Xanthomonadaceae*	*(Gamma-) Proteobacteria*
GU327738	*Yersinia entomophaga* strain MH96	*Enterobacteriaceae*	*(Gamma-) Proteobacteria*
KJ123738	*Clavibacter michiganensis subsp. michiganensis*	*Microbacteriaceae*	*(Gamma-) Proteobacteria*
KJ418436	*Clavibacter sp.* C55	*Microbacteriaceae*	*(Gamma-) Proteobacteria*
AY492242	*Corynebacterium accolens* strain CIP 104783	*Corynebacteriaceae*	*(Gamma-) Proteobacteria*
AY492252	*Corynebacterium durum* strain CIP 105490	*Corynebacteriaceae*	*(Gamma-) Proteobacteria*
AY492269	*Corynebacterium terpenotabidum* strain CIP 105927	*Corynebacteriaceae*	*(Gamma-) Proteobacteria*
AB828262	*Corynebacterium ulcerans*	*Corynebacteriaceae*	*(Gamma-) Proteobacteria*
AY859692	*Mycobacterium bolletii* CIP 108541	*Mycobacteriaceae*	*Actinobacteria*
JQ717032	*Mycobacterium kyorinense* strain KUM 60204	*Mycobacteriaceae*	*Actinobacteria*
JX303307	*Mycobacterium tuberculosis* strain 14641	*Mycobacteriaceae*	*Actinobacteria*
AY623104	*Nonomuraea sp.* ATCC 39727 rifampicin-resistant	*Strepto-sporangiaceae*	*Actinobacteria*
KC433661	*Propionibacterium acnes* strain 20089446	*Propioni-bacteriaceae*	*Actinobacteria*
KM507347	*Propionibacterium sp.* NTS31307302	*Propionibacteriaceae*	*Actinobacteria*
AY492286	*Rhodococcus equi* strain CIP 54.72	*Rhodococcaceae*	*Actinobacteria*
NC3888	*Streptomyces coelicolor* A3	*Streptomycetaceae*	*Actinobacteria*
AY492287	*Turicella otitidis* strain CIP 104075	*Corynebacteriaceae*	*Actinobacteria*
AY652737	*Alistipes finegoldii*	*Rikenellaceae*	*Bacteroidetes*
AY652738	*Alistipes putredinis*	*Rikenellaceae*	*Bacteroidetes*
KF186943	*Alloprevotella rava* strain HJX072	*Prevotellaceae*	*Bacteroidetes*
AY652741	*Bacteroides fragilis*	*Bacteroidaceae*	*Bacteroidetes*
AY652743	*Bacteroides vulgatus*	*Bacteroidaceae*	*Bacteroidetes*
KT149273	*Bacteroidetes* bacterium wkB180	*Bacteroidaceae*	*Bacteroidetes*
KF186942	Candidatus Prevotella conceptionensis strain HJM046-2	*Prevotellaceae*	*Bacteroidetes*
AY643071	*Capnocytophaga canimorsus* strain 24231	*Flavobacteriaceae*	*Bacteroidetes*
AY643070	*Capnocytophaga cynodegmi* strain CIP 103937	*Flavobacteriaceae*	*Bacteroidetes*
JX293171	*Chryseobacterium haifense* strain DSM 19056	*Flavobacteriaceae*	*Bacteroidetes*
JX293170	*Chryseobacterium koreense* strain CCUG 49689	*Flavobacteriaceae*	*Bacteroidetes*
JX293172	*Flavobacteriaceae* bacterium JJC	*Flavobacteriaceae*	*Bacteroidetes*

Table S5.3 continued.

Accession number	Bacterial strain	Family	Phyla
JX480864	*Prevotella bivia* strain ATCC 29303	*Prevotellaceae*	*Bacteroidetes*
JX480866	*Prevotella disiens* strain CCUG 9558	*Prevotellaceae*	*Bacteroidetes*
KF186918	*Prevotella multiformis* strain HJH29	*Prevotellaceae*	*Bacteroidetes*
AY826975	*Chlamydia trachomatis*	*Chlamydiaceae*	*Chlamydiae*
JN795398	*Chlamydia trachomatis* L2c	*Chlamydiaceae*	*Chlamydiae*
AY826976	*Chlamydophila psittaci* 6BC	*Chlamydiaceae*	*Chlamydiae*
JN201879	*Criblamydia sequanensis*	*Criblamydiaceae*	*Chlamydiae*
JN201886	*Estrella lausannensis*	*Criblamydiaceae*	*Chlamydiae*
KC514617	*Neochlamydia hartmannellae* strain ATCC 50802	*Parachlamydiaceae*	*Chlamydiae*
KC514603	*Protochlamydia naegleriophila* strain KNIC	*Parachlamydiaceae*	*Chlamydiae*
AF535173	*Abiotrophia defectiva* strain CIP 103242	*Aerococcaceae*	*Firmicutes*
AF172323	*Bacillus licheniformis*	*Bacillaceae*	*Firmicutes*
KF734907	*Bacillus pumilus* strain Jo2	*Bacillaceae*	*Firmicutes*
JF812023	*Bacillus sp.* 15.4	*Bacillaceae*	*Firmicutes*
JN560159	*Bacillus subtilis* strain ZZ120	*Bacillaceae*	*Firmicutes*
JF811949	*Exiguobacterium* sp. 11	*Family XII. Incertae Sedis*	*Firmicutes*
EU484367	*Geobacillus lituanicus* strain BCRC 17562	*Bacillaceae*	*Firmicutes*
EU484373	*Geobacillus thermoglucosidasius* strain BCRC 14687	*Bacillaceae*	*Firmicutes*
EU484368	*Geobacillus vulcani* strain BCRC 17563	*Bacillaceae*	*Firmicutes*
AY142827	*Heliobacillus mobilis*	*Heliobacteriaceae*	*Firmicutes*
JX398143	*Lactobacillus brevis* strain BSO 464	*Lactobacillaceae*	*Firmicutes*
EU038245	*Lactobacillus reuteri*	*Lactobacillaceae*	*Firmicutes*
GQ499852	*Paenibacillus elgii* strain NBRC 100335	*Paenibacillaceae*	*Firmicutes*
GQ499853	*Paenibacillus koreensis* strain KCTC 2393	*Paenibacillaceae*	*Firmicutes*
KF819812	*Paenibacillus sp.* Br	*Paenibacillaceae*	*Firmicutes*
KC707777	*Staphylococcus aureus* strain BP11022	*Staphylococcaceae*	*Firmicutes*
KC560772	*Streptococcus iniae* strain ISET0901	*Streptococcaceae*	*Firmicutes*
EF185159	*Veillonella atypica* strain ATCC 17744	*Veillonellaceae*	*Firmicutes*
EF185161	*Veillonella dispar* strain ATCC 17748	*Veillonellaceae*	*Firmicutes*
EF185158	*Veillonella parvula* strain ATCC 10790	*Veillonellaceae*	*Firmicutes*
GQ274966	*Fusobacterium hwasookii* ChDC F128	*Fusobacteriaceae*	*Fusobacteria*
GQ274990	*Fusobacterium periodonticum* strain ATCC 33693	*Fusobacteriaceae*	*Fusobacteria*
JX512649	*Fusobacterium simiae* strain CCUG 16798	*Fusobacteriaceae*	*Fusobacteria*
AF150880	*Leptospira biflexa*	*Leptospiraceae*	*Spirochaetes*
KJ152440	*Leptospira santarosai* strain UW	*Leptospiraceae*	*Spirochaetes*
KJ701604	*Leptospira sp.* GIMC2001:Bairam-Ali clone peg.3908	*Leptospiraceae*	*Spirochaetes*
JN935841	*Acholeplasma vituli* strain FC 097-2	*Acholeplasmataceae*	*Tenericutes*
HQ846200	*Mycoplasma iowae* serotype I	*Mycoplasmataceae*	*Tenericutes*

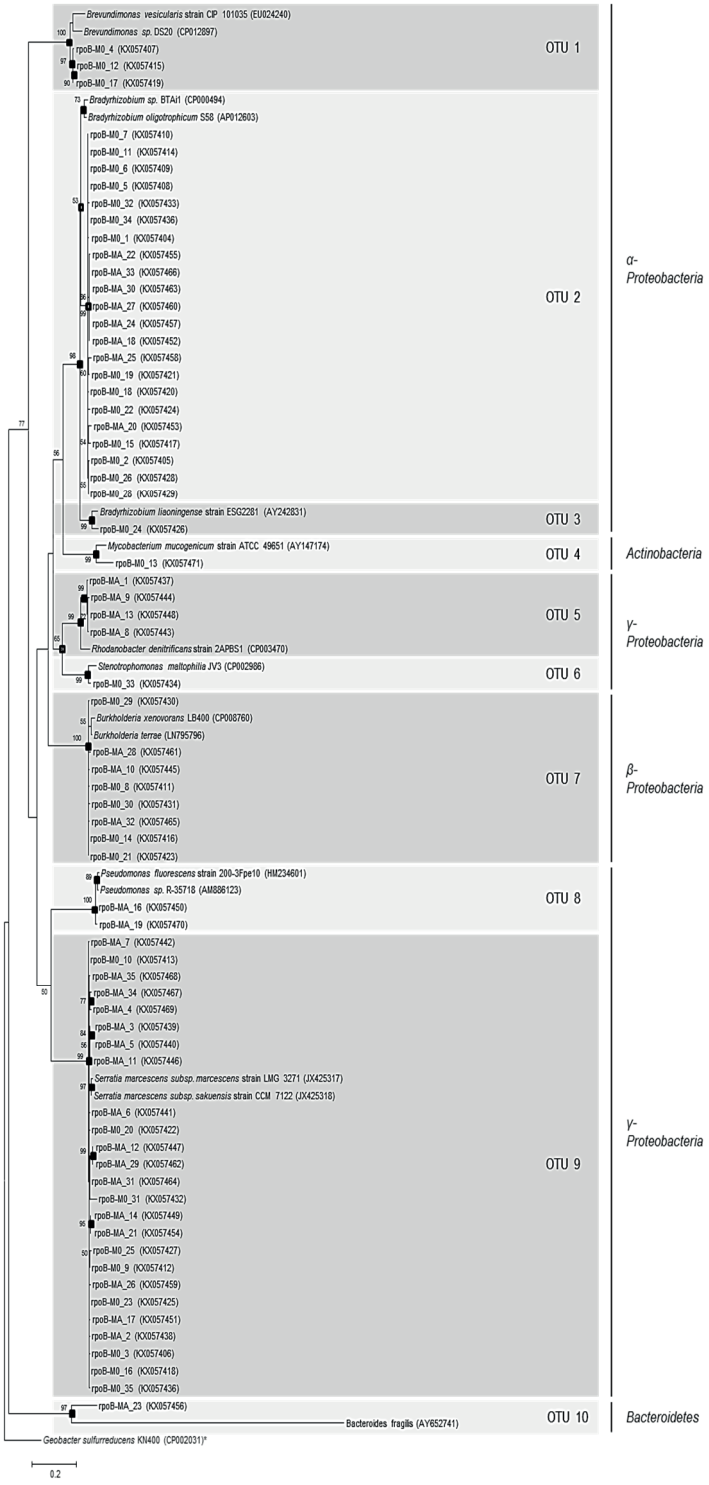

Figure S5.2. Maximum-Likelihood tree of rpoB gene sequences of bacteria associated to *M. hiemalis*. Sequences were retrieved from original (M0) and antibiotic-treated (MA) M. hiemalis isolates. Numbers next to the branches represent the percentages of replicate trees (> 50 %) in which the associated taxa clustered together in the bootstrap test (10,000 bootstraps). Tree topology was confirmed by Maximum-Parsimony and Neighbor-Joining calculations with the same dataset. Thereby, filled squares indicate similar topologies for all calculations (bootstraps > 70 %) and open squares only for Maximum-Likelihood and Neighbor-joining calculations (bootstrap > 70 %). Accession numbers are indicated in parentheses.

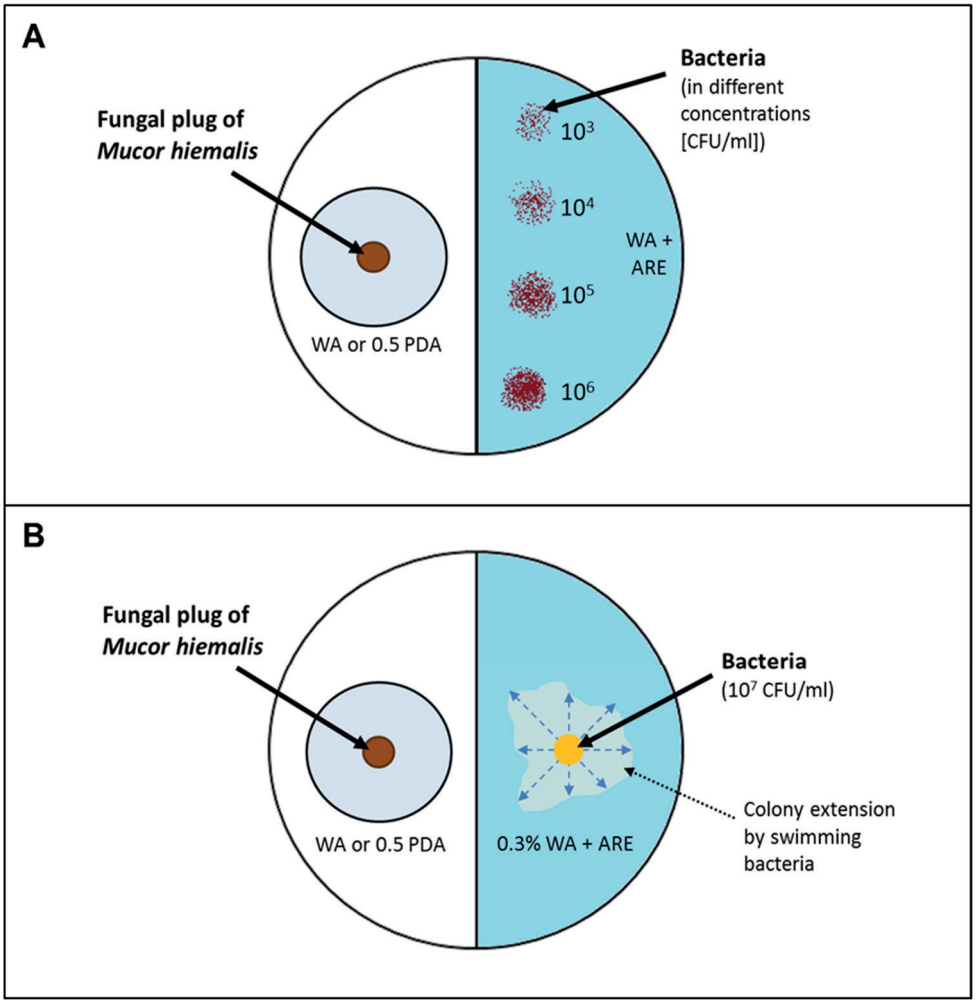

Figure S5.3. Experimental design to test the effect of fungal volatiles on rhizobacterial isolates. The bacteria *Burkholderia sp.*AD024 or *Collimonas fungivorans* Ter 331 incubated on water-agar (WA) supplied with artificial root exudates (ARE) were exposed to volatiles of *M. hiemalis* isolates growing on WA or 0.5 Potato dextrose agar (PDA). Bacterial growth was measured by counting the number of colonies for the lowest dilution of bacterial suspension added on the agar (A). The swimming motility of the bacteria was analyzed by measuring the diameter (indicated with blue arrows) of the colony at different spots (B).

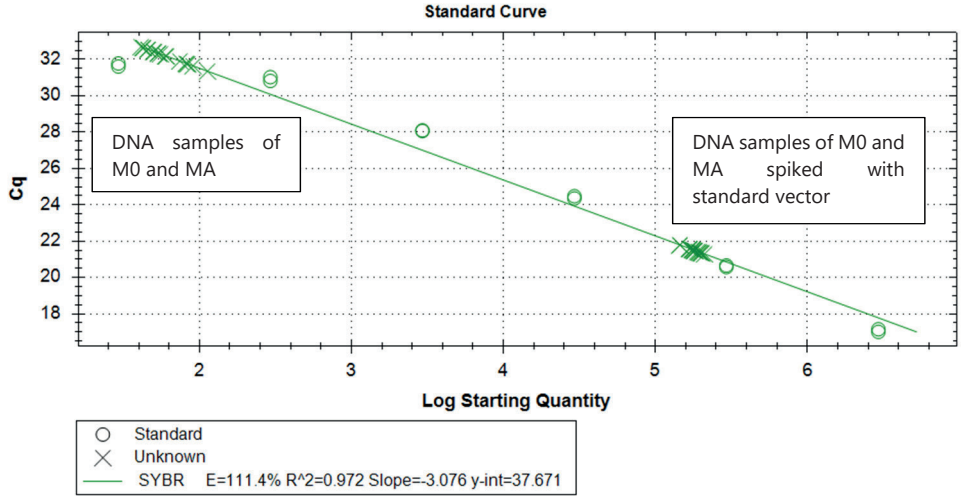

Figure S5.4. Standard curve of rpoB qPCR with DNA extracted from the original (M0) and antibiotic-treated (MA) Mucor isolate. DNA samples of were spiked (1:1) with the standard vector to correct for any inhibition.

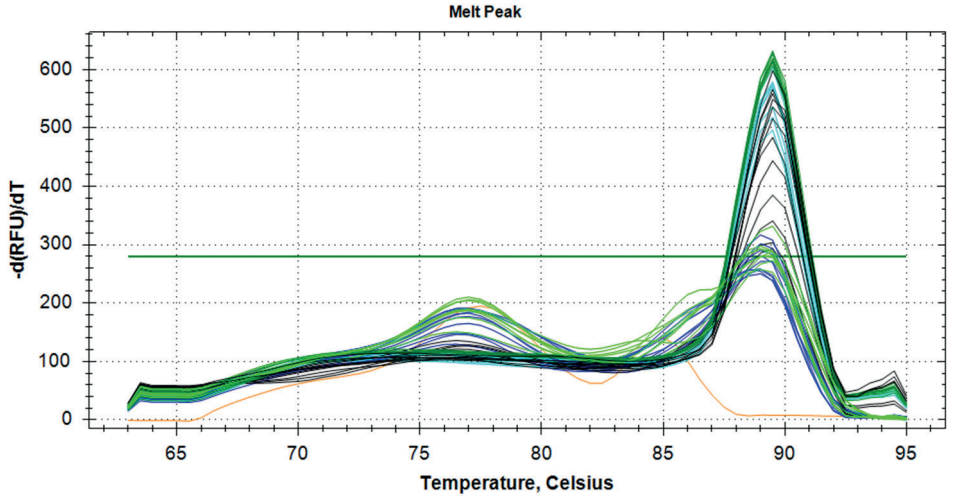

Figure S5.5. Melt curve of rpoB qPCR. Displayed are the melting curved for the standard dilutions (black), qPCR products obtained from DNA of M0 (green) and MA (blue) as well as the no template control (orange).

CHAPTER 6

Competitive Strength of Bacteria in Presence of Fungi in the Rhizosphere

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To be submitted

Abstract

Bacteria have developed various strategies to compete successfully in the rhizosphere in the presence of root-exudate consuming fungi. Many of those competitive strategies were studied under artificial lab conditions and mostly in pairwise interactions. Here we used a soil model system that more closely reflects natural conditions in the rhizosphere and applied transcriptomics and volatolomics to study mechanisms that link to the differential performance of rhizobacteria in the presence of the fungus *Fusarium culmorum*. The rhizobacteria were part of a synthetic community composed of six different bacterial species previously isolated from the rhizosphere of sand sedge (*Carex arenaria*). The abundance of the bacteria *Collimonas pratensis* and *Dyella sp.* significantly increased in fungal presence. This was correlated with the up-regulation of genes involved in the production of secondary metabolites (e.g. nonribosomal peptides, polyketides and terpenes) and defense mechanisms (e.g. multidrug exporter and aromatic compound degradation) by those bacteria. In addition, the volatile blend produced by the bacteria in fungal presence was characterized by an increase of terpenes and decrease of aromatic compounds compared to fungal absence. The abundance of *Burkholderia sp.*, *Paenibacillus sp.*, and *Pseudomonas sp.* was significantly decreased when incubated in the soil system in fungal presence. This could be linked to an up-regulation of genes related to osmotic stress and repression of defense mechanisms as well as down-regulation of secondary metabolite production. In case of *Paenibacillus sp.*, genes involved in spore formation were up-regulated and in case of *Janthinobacterium sp.* an up-regulation of genes related to chemotaxis and motility occurred in fungal presence.

Overall, this study revealed that the increased competitive strength of the bacteria *Collimonas pratensis* and *Dyella sp.* in fungal presence was linked to the acquisition of fungus-derived-nutrients (i.e. mycophagy) in combination with the induction of secondary metabolite production and defense mechanisms.

Keywords:

bacterial-fungal interactions, synthetic microbial communities, volatiles, soil microcosms

Introduction

The rhizosphere, defined as the narrow zone of soil surrounding and influenced by plant roots, is densely populated by a large number of various microorganisms (Buée *et al.* 2009, Mendes *et al.* 2013, Philippot *et al.* 2013). It was long assumed that easy degradable, low molecular weight root-exudates like sugars, amino and organic acids are mainly consumed by bacteria (De Boer *et al.* 2005). Therefore, many studies on the role of the microbial community in the rhizosphere were primarily focused on bacteria. Most rhizobacterial species are organotrophic with overlapping substrate metabolizing profiles (Strickland *et al.* 2009) and regularly compete with other bacteria for the same available nutrient source affecting the rhizobacterial community composition.

Organotrophic (saprotrophic) fungi can also metabolize and respond rapidly to rhizodeposits and make up a significant part of the microbial community in the rhizosphere (Butler *et al.* 2003, Treonis *et al.* 2004, De Boer *et al.* 2005, Denef *et al.* 2007, Paterson *et al.* 2007, Hannula *et al.* 2012). Accordingly, the composition of the rhizobacterial community can be strongly influenced by the presence of those fungi. For example, De Boer *et al.* (2015) showed that in the presence of the saprotrophic fungi *Mucor hiemalis* or *Trichoderma harzianum* in the rhizosphere of *Carex arenaria* various bacteria such as *Achromobacter*, *Burkholderia*, *Dyella*, and *Stenotrophomonas* became dominant whereas other species like *Arthrobacter*, *Pseudomonas*, and *Rhizobium* were rather abundant in fungal absence. Similarly, for the rhizosphere of sugar beet bacteria related to *Burkholderiaceae* and *Oxalobacteriaceae* became dominant in fungal presence whereby bacteria belonging to e.g. *Bradyrhizobiaceae* and *Rhizobiaceae* were more abundant in fungal absence (Chapelle *et al.* 2016). Thus, rhizobacteria must have developed adequate strategies to outcompete other bacteria successfully in fungal presence or absence.

Besides exploitative competitive strategies like fast growth and high affinity uptake systems, interference competitive strategies such as the production of growth-suppressing secondary metabolites including antibiotics, toxins, bacteriocins, biosurfactants and antimicrobial volatiles as well as the secretion of lytic enzymes are a general traits for rhizobacteria (Riley & Wertz 2002, Raaijmakers *et al.* 2009, Buée *et al.* 2009, Hibbing *et al.* 2010, Mendes *et al.* 2013). Many of these properties were extensively studied under artificial conditions like growth on nutrient rich agar and many pairwise interactions assays were performed to screen for potential biocontrol bacteria against phytopathogenic fungi (De Boer 2017). However, bacteria showing antifungal activity under lab conditions might not be competitive under natural conditions (Hol *et al.* 2013, Velivelli *et al.* 2014). It was demonstrated that interspecific interactions between non-antagonistic bacteria can trigger or suppress the production of bioactive compounds (Garbeva & De Boer 2009, Traxler *et al.*

2013 Garbeva *et al.* 2014a, Tyc *et al.* 2014, Tyc *et al.* 2017a). Furthermore, culture conditions including the supplied nutrient source can have a profound effect on the composition of produced secondary metabolite (Weise *et al.* 2012, Garbeva *et al.* 2014b) and, subsequently, on the performance of the bacteria.

The aim of this study is to gain a better understanding on bacterial strategies used to compete successfully in the presence or absence of root-exudate consuming fungi in the rhizosphere. For this purpose, we performed soil microcosm experiments with a synthetic community of six phylogenetically different rhizobacteria and the soil-borne fungus *Fusarium culmorum*. The bacteria and the fungus were previously isolated from the rhizosphere of *Carex arenaria* and incubated in soil supplied with artificial root exudates. Hence, the soil model system used in this study more closely reflects natural conditions in the rhizosphere. In addition, in contrast to metatranscriptomic studies of unknown large microbial soil communities, the system allows to easily study traits of bacteria leading to a different competitive strength in a semi-complex community with the presence of fungi. Here, we applied transcriptomics and volatolomics in fungal presence or absence to reveal the mechanisms linking to the differential bacterial performance.

Material and Methods

Bacteria, fungi, and culture media

All bacterial strains including *Burkholderia* sp. AD24, *Collimonas pratensis* Ter91, *Dyella* sp. AD56, *Janthinobacterium* sp. AD80, *Paenibacillus* sp. AD87, and *Pseudomonas* sp. AD21 and the fungus *Fusarium culmorum* PV were previously isolated from the rhizosphere of *Carex arenaria* of different dune soils (Table S6.1) and cultured as described by Schulz-Bohm *et al.* (2015) and Schulz-Bohm *et al.* (2017a). The selection of those bacteria was based on differences in phylogeny (Table S6.1) as well as a similar growth and respiration activity when incubated in rhizospheric soil, except for *Paenibacillus sp*. (Figure S6.1 A). In addition, selected bacteria possess distinct antimicrobial traits (Table S6.1 and Figure S6.1 B). Fungal spores were obtained as described by Schulz-Bohm *et al.* (2017b).

Rhizospheric soil microcosms

Rhizospheric soil microcosms were established with a synthetic bacterial community consisting of the phylogenetic different rhizobacteria (see above). The rhizobacteria were incubated in the presence or absence of the fungus *F. culmorum* PV. For the fungal

treatments (Figure S6.2), gamma-sterilized sandy soil (Schulz-Bohm *et al.* 2015) was mixed with 1.3 x 10^6 spores per g soil of *F. culmorum* and 0.18 mg glucose per g soil and pre-incubated for 5 days to induce mycelia growth. For the treatments without fungus, soil was mixed with 10 mM P-buffer (KH_2PO_4, pH 6.5) and demi-water. 10.5 g of fungus-infected or non-infected soil was mixed with 50 g gamma-sterilized sandy soil, artificial root exudates solution (ARE [Schulz-Bohm *et al.* 2015]), P-buffer and/ or bacteria with about 1 x 10^5 CFU per g soil per strain (Figure S6.2). Prior the addition of the bacterial synthetic community, overnight cultures of the bacteria were washed two-times with P-buffer (centrifugation at 5,500 rpm and 18°C) and the OD_{600} was adjusted to obtain 10^8 CFU ml^{-1}. Control treatments consisted of gamma-sterilized soil, P-buffer and ARE. The initial amount of supplied carbon of 187 µg per g soil was adjusted to the estimated range of the daily carbon inputs by roots of 100-1500 µg carbon per g rhizosphere soil (Trofymow *et al.* 1987, Cheng *et al.* 1996). The moisture content was 6.4 % (w/w). Soil microcosms were incubated at 20°C in darkness.

Fungal biomass

Biomass of the fungus *F. culmorum* PV growing in the presence or absence of bacteria in rhizospheric soil microcosms was determined by quantification of ergosterol, a sterol of the fungal cell wall. Ergosterol was extracted from soil and analyzed by HPLC according to the protocol described by De Ridder-Duine *et al.* (2006).

Direct interaction assay between bacteria and *F. culmorum*

A fungal plug (1 cm diameter) with *F. culmorum* pre-grown on 0.5 PDA was transferred to the top of a Petri-dish which was filled with 20 ml WA + ARE (Schulz-Bohm *et al.* 2016). After 24 h incubation at 25°C, 50 µl of a bacterial suspension consistent of 10^8 CFU ml^{-1} was spread out in the middle of the Petri-dish (Schulz-Bohm *et al.* 2016). The bacterial suspension was prepared as described above. Petri-dishes were closed with parafilm and incubated for 7 days at 20°C in darkness. Pictures of agar plates were taken with a Panasonic DMC-FZ200 digital camera. Fungal hyphae were observed and visualized with Leica MD641 stereomicroscope and Leica Application Suite Version 4.7.0 (Leica Microsystems CMS GmbH, Switzerland).

CO_2-production and growth of bacteria in soil supplied with artificial root exudated (ARE)

Incubation conditions of the bacteria in soil supplied with ARE as well as the determination of CO_2-emission and CFU number were described previously (Schulz-Bohm *et al.* 2015).

Siderophore activity bioassay

Bacteria were incubated for 5 days on water-yeast-agar (Garbeva & De Boer 2009) followed by an overlay agar mixed with ChromoAzurolS agar as described by Perez-Miranda *et al.* 2007.

Collection and analysis of volatiles emitted from soil microcosms

Rhizospheric soil microcosms were set up as described above in glass petri-dishes with an exit at the top to which a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd., Llantrisant, UK) could be fixed. Microbial and soil volatile organic compounds (VOCs) were trapped for 12h after 3.5 or 5.5 days of incubation. All treatments were incubated in five replicates. Traps were removed, capped and stored at 4°C until analysis by GC-MS (Schulz-Bohm *et al.* 2017b).

Nucleic acid extraction and quantitative PCR (qPCR)

Nucleic acids were extracted from 0.5-0.85 g soil according to Schulz-Bohm *et al.* (2015). The extracts were stored at -80°C or subsequently used for qPCR or DNAse treatment. To determine the abundance of bacteria in the different treatments qPCR of the 16S rRNA gene was performed with a BioRad CFX96 C1000 TouchTM Thermal cycler. The 16S rRNA genes of bacterial strains were quantified according to the protocols as described by Schulz-Bohm *et al.* (2015) and Schulz-Bohm *et al.* (2017b).

DNase treatment and RNA sequencing

Nucleic acids extracted from about 0.8 g soil per sample were treated according to the manufacturer's protocol with 25 U HC DNase I (Thermo Fisher Scientific, the Netherlands) and incubated for 45 min at 37°C. The RNA was purified by an overnight precipitation with ammonium acetate and ethanol as described before (Schulz-Bohm *et al.* 2015). Purified RNA extracts of four extractions per sample were pooled prior overnight precipitation and stored at -80°C. Quality of the RNA was checked with a Fragment Analyzer (Advanced Analytical, Germany) and DNF-471 Standard Sensitivity RNA Analysis kit (Advanced Analytical, Germany) according to the manufacturer's protocol. Further RNA preparation as well as RNA sequencing was performed at ErasmusMC Center for Biomics (Rotterdam, the Netherlands). RNA preparation was conducted with ScriptSeq Complete kit for bacteria (Illumina, the Netherlands) consisting of a Ribo-Zero Bacteria rRNA removal step and ScriptSeq v2 RNA-Seq kit (Illumina) for sequencing library preparation. RNA sequencing

(100 bp length per read) was performed according to the Illumina TruSeq v3 protocol on the HiSeq2500 (Rapid mode) for SR100bp + 7bp index.

Transcriptome analysis

Transciptome analysis was performed with the workflow engine snakemake (Köster & Rahmann 2012) in combination with Trinity (Grabherr *et al.* 2011). Within this pipeline, obtained RNA-sequencing reads were filtered using Fastq MCF (Aronesty 2011) and aligned against the annotated genome sequence (see below) of each bacterium and *F. culmorum* with Bowtie 2 (Langmead & Salzberg 2012). Normalization of transcriptome data (in regard to differences in number of reads and abundance of transcripts per sample gained from different treatments) and estimation of transcript abundance was performed with RSEM V1.1.26 (Li & Dewey 2011) and differential expressed genes were analyzed by using edgeR V3.2 (Robinson *et al.* 2010; McCarthy *et al.* 2012) and DESeq2 Version 1.8.2 (Love *et al.* 2014). Data were filtered with a P value of < 0.025 and a FDR value < 0.05.

Genome sequencing, assembly and annotation

Annotated genomes of *C. pratensis* Ter91 (Song *et al.* 2015b), *Burkholderia sp.* AD24 (Tyc et al. 2017a), *Paenibacillus sp.* AD87 (Tyc et al. 2017a) as well as *F. culmorum* PV (Schmidt 2017) were available before. For Dyella sp. AD56, Janthinobacterium sp., and Pseudomonas sp. AD21 genomic DNA was isolated with QIAamp DNA Mini Kit (QIAGEN , the Netherlands) according to the manufactures protocol and subsequently used for sequencing. The library preparation, genome sequencing and assembly of *Dyella sp.* AD56 and *Janthinobacterium sp.* AD80 were conducted by MicroLife Solutions B.V. (Amsterdam, the Netherlands). In brief, libraries were made with the Ion Xpress Plus Fragment Library kit (ThermoFisher), which entailed enzymatic shearing and size selection on a E-Gel® SizeSelect TM 2% Agarose Gel (ThermoFisher). All intermediate steps were monitored on a QUBIT fluorometer and 2100 Bioanalyzer (Agilent). 10 pM of these libraries were used to make sequencing templates with the IonChef system (ThermoFisher). In parallel, also a mate-pair approach was deployed with the same DNA samples obtained from *Dyella sp.* AD56 and *Janthinobacterium sp.* AD80, by first sonicating the genomic DNA to fragments of approximately 8 kbp in a Branson2510 sonificator at 60°C. This was used for the library preparation with the Ion TrueMate Library preparation kit which was then amplified and selected on size, prior to the template preparation. Sequencing of all libraries was performed with the Ion PGM™ Hi-Q™ Sequencing Kit (ThermoFisher) obtaining 328543 and 425620 of 200 bp single end reads as well as 116830 and 672906 400 bp mate-pair reads for *Dyella sp.* AD56 and

Janthinobacterium sp. AD80, respectively. For each strain, single end reads were first assembled with MIRA version 4.0 (Chevreux *et al.* 2004) and in combination with BESST scaffolder (Sahlin *et al.* 2014) contigs scaffolds were made based on the information provided by the mate-pair approach.

Genome sequencing and quality analysis of *Pseudomonas sp*. AD21 was performed by BaseClear Group (Leiden, the Netherlands) via an Illumina platform with an average read length of 250 bp and Illumina Casava pipeline version 1.8.0. The Illumina reads were filtered using Fastq MCF with default settings and assembled using SPAdes 3.8.0 (Bankevich *et al.* 2012). The genome assembly properties of *Dyella sp.* AD56, *Janthinobacterium sp.* AD80, and *Pseudomonas sp.* AD21, are presented in Table S6.2. The annotation of final contigs for all bacteria were performed with a modified version of PROKKA V1.11 (Seemann 2014) and COG annotations were obtained as described by Tyc *et al.* (2017a) and manually adapted according to available descriptions of gene functions. Annotation of the *F. culmorum* genome was available from a previous study (Schmidt 2017). *In silico* analysis of secondary gene clusters (Table S6.3) was performed with antiSMASH 4.0.2 (https://fast.antismash.secondarymetabolites.org, last visit: 09-10-17). Draft genomes of *Pseudomonas sp.* AD21, *Dyella sp*. AD56, and *Janthinobacterium sp*. AD80 were submitted to the NCBI genome database (Accession number: NQYG00000000, NRDP00000000, and NRDQ00000000).

Statistical analysis

Multivariate analysis of processed and normalized (log transformation and mean centered) GC-MS data was conducted with MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/MetaboAnalyst, Xia *et al.* 2015). Compounds were accounted as produced for each treatment when the average peak intensity for all replicates per treatment was at least 2-fold higher and significant different (Student's T-test, $P < 0.05$) from the controls.

The statistical analysis on qPCR data and the fungal biomass based on the quantification of ergosterol was performed with R 3.1.1 (http://www.r-project.org/) using one-way Anova followed by Tukey's HSD test (De Mendiburu 2014) or Student's T-test. To obtain normality of errors, data were log-transformed. Differences revealed by statistical tests were considered significant for $P < 0.05$.

Results

Bacterial performance in fungal absence and presence

A synthetic community consisting of six phylogenetic different rhizobacteria was incubated in the presence or absence of the fungus *Fusarium culmorum* in soil supplemented with root exudates (Figure S6.2). Within six days of incubation, the bacterial abundance increased from about 1 x 10^5 cells per g soil to maximal 1 x 10^9 and 8 x 10^8 cells per g soil in fungal absences and presence, respectively (Figure 6.1). *Burkholderia sp*., *Janthinobacterium sp*., and *Pseudomonas sp*. were the most abundant bacterial strains in both treatments (> 2 x 10^8 cells per g soil). However, in presence of *F. culmorum* the abundance of those bacteria including *Paenibacillus sp*. was significant lower as compared to the treatment without *F. culmorum* (Figure 6.1). In contrast, the abundance of *Collimonas pratensis* and *Dyella sp*. was significant higher in fungal presence (6 x 10^6 and 2 x 10^7 cells per g soil with fungus, respectively, versus 3 x 10^6 and 8 x 10^6 cells per g soil without fungus, respectively).

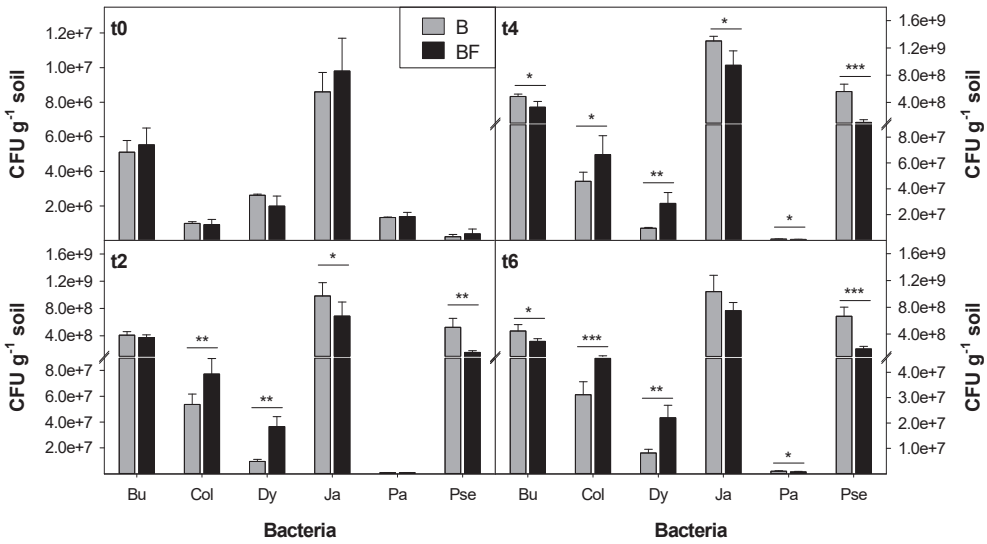

Figure 6.1. Bacterial abundance in fungal presence (BF) or absence (B). A synthetic community consisting of the bacteria *Burkholderia sp.* AD24 (Bu), *Collimonas pratensis* Ter91 (Col), *Dyella sp.* AD56 (Dy), *Janthinobacterium sp*. AD80 (Ja), *Paenibacillus sp.* AD87 (Pa), and *Pseudomonas sp.* AD21 (Pse) was incubated in presence or absence of the fungus *F. culmorum* PV in rhizospheric soil for 6 days and quantified by qPCR every two days (t0-t6). Asterisks indicate per bacterium significant differences among the treatments with * $p < 0.05$, **$p < 0.01$ and *** $p < 0.001$.

Fungal growth in the presence or absence of bacteria

The biomass of *F. culmorum* based on the quantification of fungal ergosterol increased four to five times within six days of incubation in rhizospheric soil (Figure 6.2). In presence of the bacterial synthetic community, the fungal biomass was significantly decreased as compared to the treatment without bacteria (1.1 mg ergosterol per kg soil with bacteria and 1.6 mg ergosterol per kg soil without bacteria; Figure 6.2). This suggests that the growth of *F. culmorum* is affected by competitive interactions with the bacteria. Direct interaction assays on water-agar supplied with ARE demonstrated that the bacteria *Collimonas pratensis* and *Janthinobacterium sp*. but not *Dyella sp*. were able to inhibit hyphal extension of *F. culmorum* (Figure S6.3). Furthermore, the fungal hyphae seemed to be visibly impaired by interactions with *Collimonas pratensis* and *Janthinobacterium sp*. (Figure S6.3 E and F).

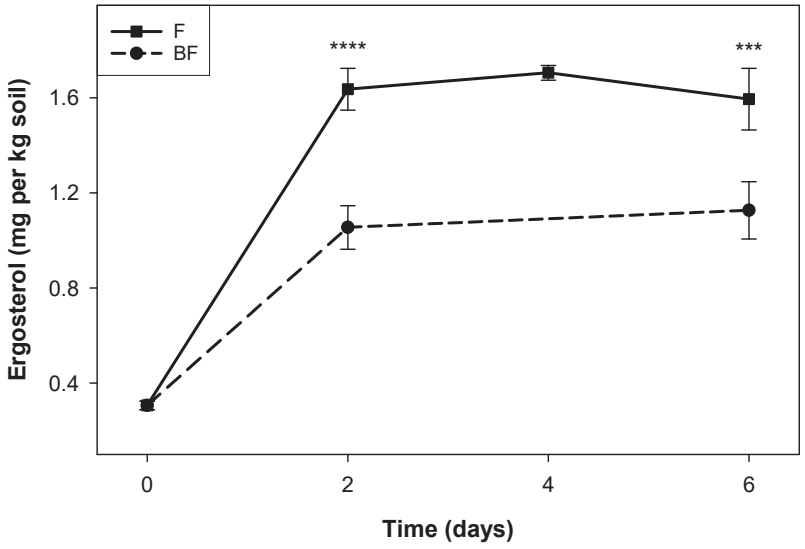

Figure 6.2. Increase of fungal biomass determined by the amount of ergosterol of *F. culmorum* PV in presence (BF) or absence (F) of bacteria in rhizospheric soil. Asterisks indicate differences among the treatments with *** $p < 0.0001$.

Differential gene expression of bacteria in interaction with *F. culmorum*

To obtain more insights into the mechanisms linking to the differential performance of the bacteria in fungal presence, RNA sequencing was performed for soil samples collected after four and six days of incubation. In total, 23-27 million reads per sample in high quality were obtained (Table S6.3 and Figure S6.4). The composition of differentially expressed genes (DEG), obtained by combined analysis with EdgeR and DESeq2, for replicates of the same treatment as well as for the incubation times of four or six days was similar (Figure S6.5). The total number of significantly up-regulated bacterial genes in fungal presence was about 1.5-times lower as compared to the number of down-regulated genes (3007 versus 4248 and 2775 versus 4178 genes for four and six days of incubation, respectively; Table S6.10-S6.13).

DEG could be assigned to 25 different orthologous gene categories (COG). However, the function of most DEG remained unknown (Figure 6.3 and Figure S6.6). In the presence of *F. culmorum*, most up-regulated genes belonged to *Collimonas pratensis* and *Dyella* sp. and especially to the COG classes "Amino acid transport and metabolism", "Cell wall/ membrane/ envelope biogenesis", "Energy production and conversion", and "Translation, ribosomal structure and biogenesis" (Figure 6.3 and Figure S6.6). In contrast, many genes of *Burkholderia sp.*, *Paenibacillus sp.* and *Pseudomonas sp.* were strongly down-regulated, especially genes assigned to the COG classes "Amino acid transport and metabolism", "Carbohydrate transport and metabolism", "Transcription", "Translation, ribosomal structure and biogenesis", and "Signal transduction mechanisms" (Figure 6.3 and Figure S6.6). Besides the classification into COG, DEG were assigned to various functional groups as described hereafter and summarized in Table S6.4-S6.9.

Differentially expressed bacterial genes involved in osmoregulation and other stress responses

In the presence of *F. culmorum*, genes related to the biosynthesis and transport systems of osmo-regulatory solutes were up to 7.0 log2fold up-regulated in *Burkholderia sp.*, *Collimonas pratensis, Dyella sp., Janthinobacterium sp.* and particularly in *Pseudomonas sp.* (Figure 6.4 A and Table S6.4). Additionally, various other genes related to environmental stress responses such as carbon and phosphate limitation or oxidative stress were differentially expressed by the bacteria in the presence of *F. culmorum* (Figure 6.4 A and Table S6.4).

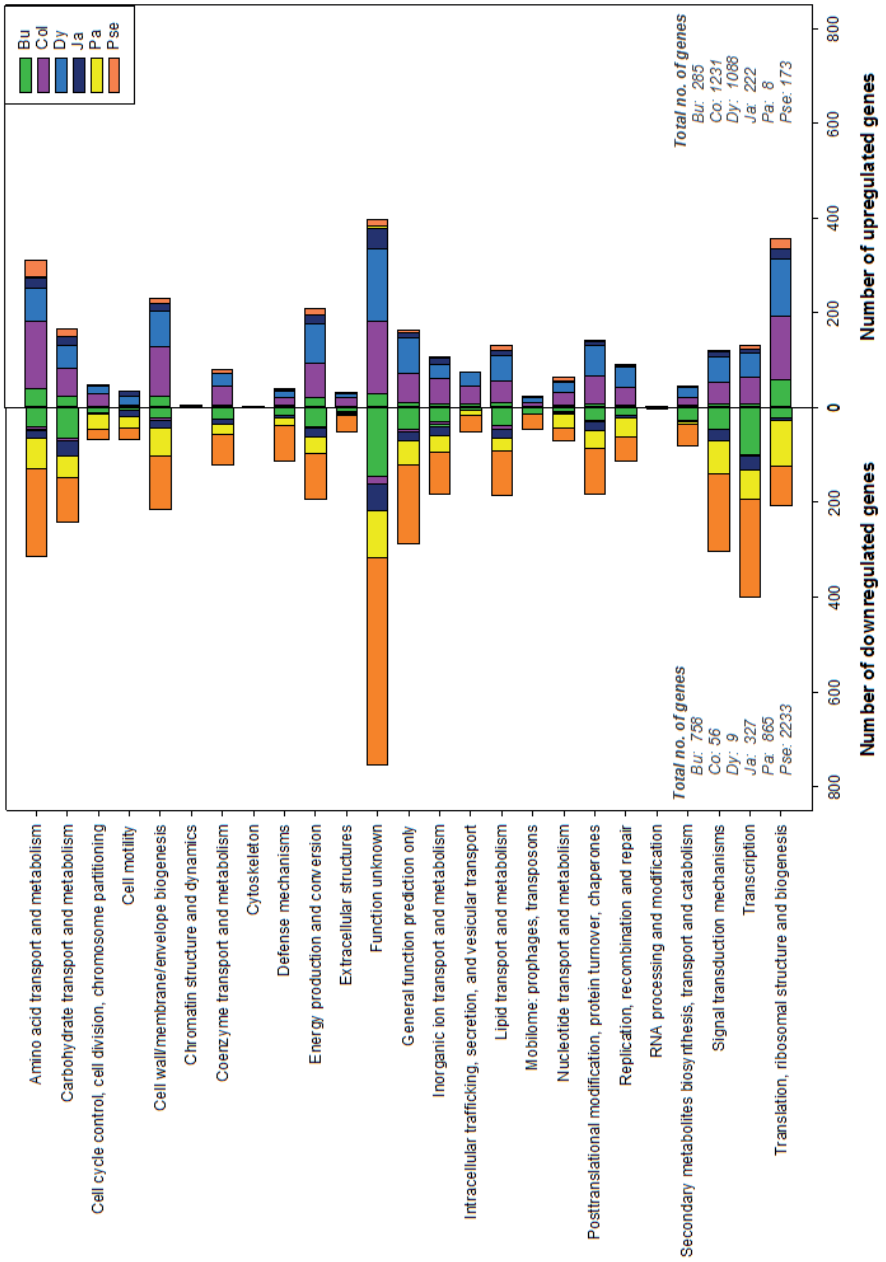

Figure 6.3. Differentially expressed genes assigned to various orthologous gene categories (y-axis) of bacteria in fungal presence or absence. A bacterial synthetic community composed of *Burkholderia sp.* AD24 (Bu), *Collimonas pratensis* Ter91 (Col), *Dyella sp.* AD56 (Dy), *Janthinobacterium sp.* AD80 (Ja), *Paenibacillus sp.* AD87 (Pa), and *Pseudomonas sp.* AD21 (Pse) was incubated for 4 days in presence or absence of the fungus *F. culmorum* PV in rhizospheric soil. Bacterial genes were accounted as up- or downregulated in the presence of the fungus with a p-value of < 0.025 and a FDR value < 0.05. Total number of up- or downregulated genes per bacteria are listed at the bottom of the figure.

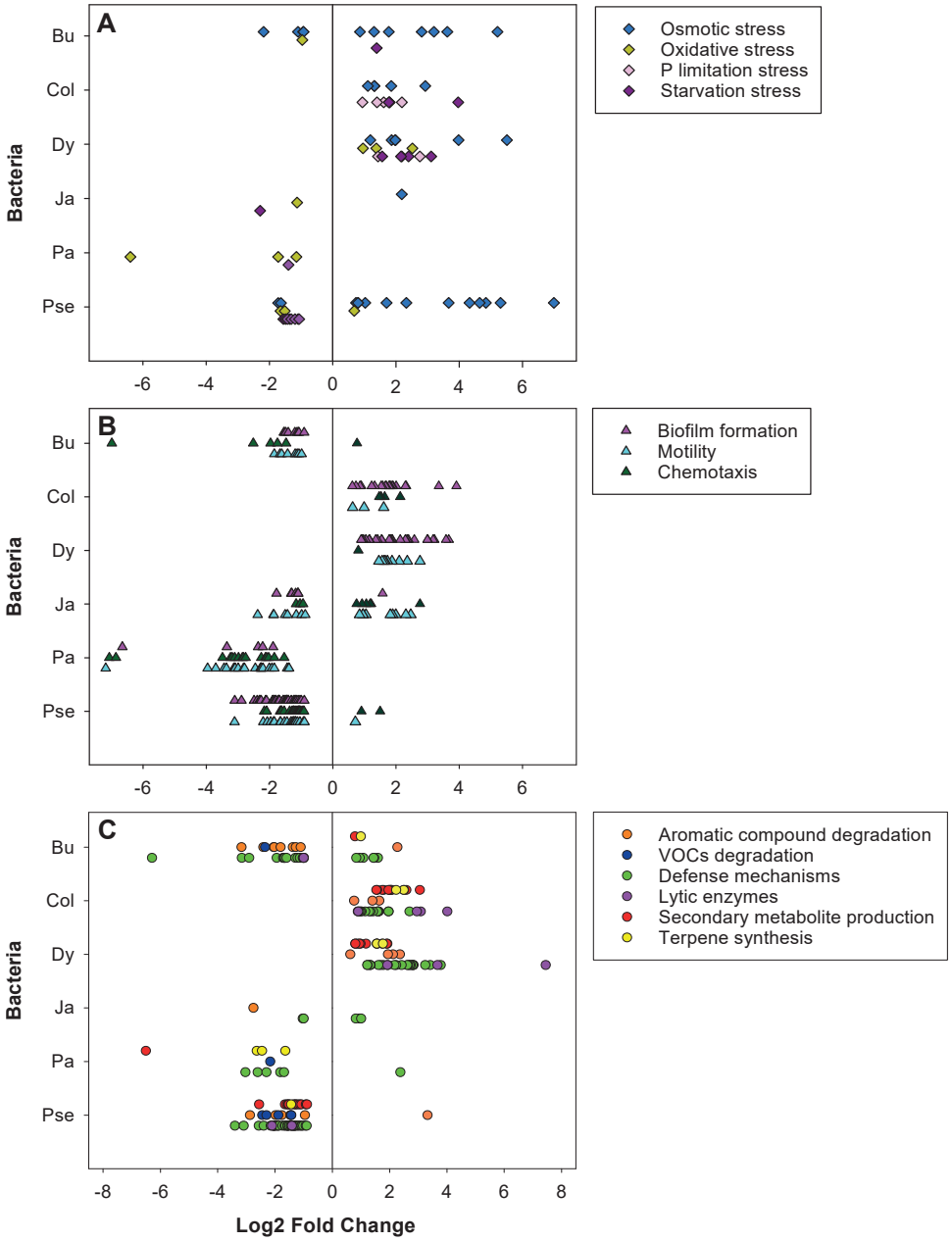

Figure 6.4. Differentially expressed bacterial genes related to different stress responses (A), biofilm formation, motility or chemotaxis (B) as well as secondary metabolite production and defense mechanisms (C). Up-regulated and down-regulated genes of bacteria incubated as synthetic community in presence of *F. culmorum* PV in rhizospheric soil are indicated by log2 fold change > 0 and < 0, respectively. Bacterial genes were account as up- or downregulated in the presence of the fungus with a p-value of < 0.025 and a FDR value < 0.05. Abbreviations: Bu, *Burkholderia sp*. AD24; Col, *Collimonas pratensis* Ter91; Dy, *Dyella sp*. AD56; Ja, *Janthinobacterium sp.* AD80; Pa, *Paenibacillus sp.* AD87; Pse, *Pseudomonas sp*. AD21.

Differentially expressed bacterial genes related to signal transduction

Several genes involved in signal transduction mechanisms were differentially expressed by the bacteria (Figure 6.3, Figure S6.6 and Table S6.10-S6.13). In the presence of *F. culmorum*, genes related to chemotaxis proteins were significantly down-regulated (in total 47 versus 14 up-regulated genes [Table S6.5]). A down-regulation of those genes was observed specifically for *Burkholderia sp.*, *Paenibacillus sp.* and *Pseudomonas sp.* (Figure 6.4 B). For *Collimonas pratensis, Dyella sp.*, and *Janthinobacterium sp.,* genes involved in chemotaxis were up-regulated in fungal presence (Figure 6.4 B and Table S6.5).

DEG involved in cell motility and biofilm formation of the bacteria

In fungal presence, bacterial genes encoding for proteins of the flagellar motor and genes involved in cell motility were mostly down-regulated, except for *Collimonas pratensis, Dyella sp.*, and *Janthinobacterium sp.* (Figure 6.4 B and Table S6.6). Furthermore, in the presence of *F. culmorum* genes related to biofilm formation were completely down-regulated in *Burkholderia sp.*, *Janthinobacterium sp.*, *Paenibacillus sp.*, and *Pseudomonas sp.* (Figure 6.4 B and Table S6.7). In case of *Collimonas pratensis* and *Dyella sp.*, the expression of those genes was induced in fungal presence (Figure 6.4 B).

Differentially expressed bacterial genes related to nitrogen and carbon uptake

Various genes encoding for enzymes, regulons, and transport systems involved in the assimilation of nitrogen were differentially expressed in fungal presence (Table S6.10-S6.13). For example, bacterial genes encoding for the glutamine synthetase and glutamate synthase as well as genes related to glutamine transporter were down-regulated in fungal presence (Table S6.8). In comparison, genes encoding for the glutamate dehydrogenase and genes related to glutamate transporter were significant up-regulated, in particular for *Burkholderia sp., Janthinobacterium sp.*, and *Collimonas pratensis* (Table S6.8). In case of *Burkholderia sp.*, *Dyella sp.* and *Collimonas pratensis*, genes related to histidine uptake and degradation such as histidine ammonia-lyase or urocanate hydratase were up-regulated in the presence of *F. culmorum* (Table S6.8).

In fungal presence, genes involved in carbohydrate transport of e.g. ribose and trehalose as well as glycerol-3-phosphate were up to 7.41 log2fold up-regulated in Burkholderia sp., Collimonas pratensis, and Pseudomonas sp. (Table S6.8). A transcription regulator in response to carnitine was up-regulated by the bacteria in fungal presence (Table S6.8).

DEG related to secondary metabolite production and defense mechanisms

Various bacterial genes involved in secondary metabolite biosynthesis, transport and catabolism were differentially expressed (Figure 6.3, Figure S6.6 and Table S6.10-S6.13). However, in fungal presence the number of down-regulated genes was two times higher as compared to up-regulated genes. In particular, genes related to the biosynthesis of nonribosomal peptides such as gramnicidin or tyrocidine, antifungal antibiotics like pyrrolnitrin, polyketides, pigments such as betalains or tetrahydrocurcumin as well as genes involved terpene metabolism were completely down-regulated in *Paenibacillus sp.* and *Pseudomonas sp.* in presence of *F. culmorum* (Figure 6.4 C and Table S6.9). In contrast, for *Dyella sp.* and *Collimonas pratensis* genes involved in secondary metabolism such as the biosynthesis of polyketides, nonribosomal peptides, bacteriocins, siderophores and terpenes were particularly up-regulated in fungal presence (Figure 6.4 C, Table 6.1 and Table S6.9). In case of degradation of aromatic compounds, related genes were up to 2.4 log2fold up-regulated in *Dyella sp.* and *Collimonas pratensis* in fungal presence while for *Burkholderia sp*. and *Pseudomonas sp*. numerous genes were down-regulated (Figure 6.4 C and Table S6.9).

Some of the DEG could be assigned to secondary gene cluster identified by *in silico* analysis of the genome sequences (Table 6.1). For *Collimonas pratensis* and *Dyella sp.* many of those gene clusters were identified to be up-regulated in fungal presence while for *Paenibacillus sp.* and *Pseudomonas sp.* genes associated to secondary gene cluster were mostly down-regulated.

For *Dyella sp.* and *Collimonas pratensis*, genes related to defense mechanisms like multidrug resistance proteins, multidrug or macrolide exporter, Type VI secretion system as well as beta-lactamase, chitinase, extracellular protease, and phospholipase C activity were especially up-regulated in the presence of *F. culmorum* (Figure 6.4 C and Table S6.9). In case of *Pseudomonas sp.*, all genes involved in defense mechanisms including the activity of antifungal proteins such as chitinase, proteases, and alkaline protease inhibitor were down-regulated in fungal presence. Similar trend was observed for *Burkholderia sp.* and *Paenibacillus sp.* (Figure 6.4 C and Table S6.9). In case of *Paenibacillus sp*., genes related to spore formation were up-regulated (Table S6.12).

Table 6.1. Overview of secondary metabolite gene clusters obtained by *in silico* analysis of the bacterial genome sequences with antiSMASH. Abbreviations: Bu, Burkholderia; Dy, Dyella; Ja, Janthinobacterium; Pa, Paenibacillus; Pse, Pseudomonas.

Gene Cluster	Bu sp. AD24	Co Ter91	Dy sp. AD56	Ja sp. AD80	Pa sp. AD87	Pse sp. AD21
1	Terpene	NRPS[A]	Aryl-polyene[A]	Indole	Terpene[B]	NRPS
2	Arylpolyene[B]	Arylpolyene[A]	Bacteriocin	Terpene	T3PKS[B]	Arylpolyene[B]
3	Bacteriocin[B]	Hser-lactone	Terpene	Bacteriocin	Siderophore	Siderophore
4	Phospho-nate[B]	Terpene	Lantipeptide-T1PKS-NRPS[A]	Bacteriocin	Other[B]	Hserlactone-Butyro-lactone[B]
5	Terpene[B]	NRPS[A]	Lanti-peptide	Arylpolyene	Lasso-peptide[B]	Bacteriocin[B]
6	Terpene[B]	Terpene[A]	Bacteriocin[A]	NRPS	Bacteriocin	NRPS[B]
7	Bacteriocin	Thio-peptide[A]	NRPS	Bacteriocin	NRPS[B]	NRPS[B]
8	Hserlactone-NRPS[B]	T1PKS-NRPS[A]	NRPS[A]	NRPS	Lantipeptide	NRPS[B]
9	Bacteriocin		NRPS[A]		Terpene[B]	NRPS[B]
10	Bacteriocin		Terpene[A]		Lantipeptide[B]	Other
11	T1PKS[B]		NRPS			NRPS
12	Hserlactone-NRPS[A]		NRPS			

[A] Bacterial genes belonging to secondary metabolite gene cluster were up-regulated in fungal presence (see Table S6.10).
[B] Bacterial genes belonging to secondary metabolite gene cluster were down-regulated in fungal presence (see Table S6.11).

Volatile production of a bacterial community and *F. culmorum* in rhizospheric microcosms

A different blend of volatile organic compounds was produced by the bacteria in fungal presence and absence as well as *F. culmorum* in soil after four and six days of incubation (Figure 6.5 A and Figure S6.7). Most volatiles were detected for the interaction of bacteria with *F. culmorum* (41-46 versus 18 and 32 for the treatments fungus and bacteria only, respectively) (Table S6.15). However, a particular amount (> 15 %) of those detected compounds could not be assigned for certainty and remained unidentified. For all treatments, many identified VOCs were ketones (Figure 6.5 B and Table S6.15). Besides ketones (38 %), volatiles released by the bacterial community were composed of mostly aromatic (16 %) such as 2-(1,1-dimethylethyl)-1,4-Benzenediol and alcoholic compounds (19 %) like 3-Heptanol (Figure 6.5 B). In contrast, in the presence of *F. culmorum*, less alcoholic and aromatic volatile compounds (12 % and 7 %, respectively) were emitted and

terpenes such as camphenilone and fenchone were produced (Figure 6.5 B and Table S6.15). VOCs emitted by *F. culmorum* were dominated by ketones (44 %) and terpenes (17 %) like α-cedrene. Furthermore, alkenes were not detected for *F. culmorum* as compared to the volatile blend produced in treatments containing the bacterial community (Figure 6.5 B and Table S6.15).

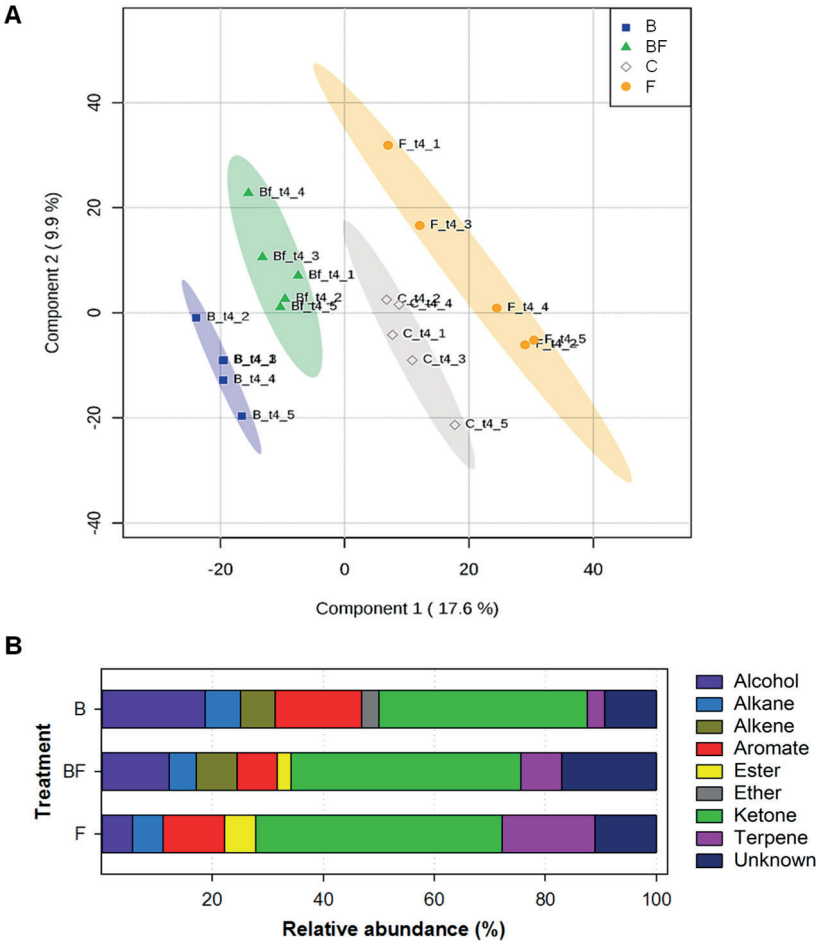

Figure 6.5. Volatile profile for *F. culmorum* PV (F) and a bacterial community incubated for 4 days in presence (BF) or absence (B) of *F. culmorum* in rhizospheric soil. The bacterial community consisted of *Burkholderia sp.* AD24, *Collimonas pratensis* Ter91, *Dyella sp.* AD56, *Janthinobacterium sp.* AD80, *Paenibacillus sp.* AD87, and *Pseudomonas sp.* AD21. A: PLS-DA score plot of volatiles produced by the different treatments. Controls (C) consisting of soil and artificial root exudates were included in the analysis. B: Relative abundance of identified volatiles (different from the control) which were assigned to various compound classes.

Discussion

Bacteria developed various strategies to compete successfully in the rhizosphere environment in the presence of root-exudate consuming fungi. Here we studied mechanisms that link to the differential performance of rhizobacteria in fungal presence (Figure 6.6) by using a soil model system that more closely reflects natural conditions in the rhizosphere.

We observed that the abundance of two bacteria, namly *Collimonas pratensis* and *Dyella sp*. significantly increased in the presence of the fungus *F. culmorum* while the abundance of the other bacteria significantly decreased. This was correlated with the total number of up-regulated and down-regulated genes per bacterium in fungal presence. Especially genes associated to secondary metabolite production including nonribosomal peptide synthases such as gramicidin or tyrocidine synthase (Felnagle *et al.* 2008), polyketide synthases and enzymes such as phytoene synthase and 1-deoxy-D-xylulose-5-phosphate synthase involved in biosynthesis of terpenoids or terpenes (Oldfield & Lin 2012; Kirby *et al.* 2015) were up-regulated in *Collimonas pratensis* and *Dyella sp*. in fungal presence. In contrast, genes related to secondary metabolite production were completely down-regulated in *Paenibacillus sp*. and *Pseudomonas sp*. in the presence of *F. culmorum*. Nonribosomal peptides, polyketides and terpenes can play important roles in interspecific competitive interactions (Mendes *et al.* 2013; Schmidt *et al.* 2015). The induced production of secondary metabolites might explain the higher competitive strength of *Collimonas pratensis* and *Dyella sp*. in fungal presence. Similarly, an induction of secondary metabolite production was reported for the interaction of *Collimonas fungivorans* with *Aspergillus niger* (Mela *et al.* 2011).

Some secondary metabolites like terpenes, specifically mono-, sesqui-, and diterpenes (Herrmann 2011), can be volatile. Volatile compounds have the ability to diffuse through air- and water-filled pores in soil (Insam & Seewald 2010; Effmert *et al.* 2012) and, therefore, they can have a wide effective range in soil. Here, we performed volatilomics to link the up-regulation of bacterial genes involved in terpene synthesis to the actual production of those compounds in the soil model system. Indeed, terpenes including camphenilone and fenchone were emitted when the bacteria were incubated in the presence of *F. culmorum*. Those terpenes might be produced by the bacteria as response to interspecific interactions. For instance, it was previously shown that the monoterpene fenchone, known to have antibacterial activity (Kazemi *et al.* 2012), can be produced by interactions of bacteria including *Dyella sp.* in soil under similar conditions (Schulz-Bohm *et al.* 2015). However, *F. culmorum* is also able to produce various volatile terpenes including fenchone or camphene as observed here and elsewhere (Schmidt *et al.* 2016; Schulz-Bohm *et al.* 2017b).

In addition, genes related to the biosynthesis of terpenes were expressed by *F. culmorum* in presence of the bacteria (Table S6.14). Thus, it is plausible that some terpenes detected for the interactions of bacteria with *F. culmorum* were not produced by the bacteria but by the fungus itself.

Besides terpenes also other volatile compounds released in interaction of bacteria and fungus such as ketones might be involved in competitive interactions of the bacteria. Furthermore, volatile compounds are proposed to have synergistic effect in combination with antibiotics (Schmidt *et al.* 2015).

In interaction of the bacteria with *F. culmorum*, less aromatic compounds were emitted. In case of *Collimonas pratensis* and *Dyella sp*., genes related to the degradation of aromatic compounds were up-regulated in fungal presence. Also for *F. culmorum*, genes involved in aromatic compound degradation were expressed in interaction with the bacteria (Table S6.14). Other than serving as potential carbon source, many aromatic compounds can have antimicrobial activity. Hence, the capacity to degrade those compounds might serve as protection for both, bacteria and fungus (Stopnisek *et al.* 2016).

In the presence of *F. culmorum*, additional defense mechanisms such as **resistance nodulation cell division efflux systems** (i.e., multidrug or macrolide exporter) were up-regulated in *Dyella sp*. and *Collimonas pratensis*. The activity of those efflux systems can contribute to the resistance against various antibiotics (Fernando & Kumar 2013) that were potentially released by the other bacteria and *F. culmorum*. Genes for the biosynthesis of antimicrobial nonribosomal peptides such as ustiloxin (Tsukui *et al.* 2015) and polyketides like fusaric acid (Niehaus *et al.* 2014) as well as other mycotoxins and antibiotics such as penicillin were expressed by *F. culmorum* in the presence of the bacteria (Table S6.14). Interestingly, genes related to defense mechanisms like multidrug exporter were completely down-regulated in *Pseudomonas sp.* and *Paenibacillus sp.* that might have contributed to a lower competitive strength in fungal presence.

Another explanation for the success of *Dyella sp*. and *Collimonas pratensis* in fungal presence might be the up-regulation of genes related to antifungal properties such as chitinase and extracellular protease (De Boer *et al.* 2015). Chitinase activity can be indicative for a mycophageous lifestyle, i.e. retrieving nutrients from living fungal hyphae (Leveau *et al.* 2010). Mycophageous activity of *Collimonas pratensis* against *F. culmorum* was confirmed by direct interaction assay on nutrient-poor agar. Also for some strains of the genus *Dyella* an association to fungal hyphae and a potential mycophageous lifestyle was reported (Warmink & van Elsas 2009; Rudnick *et al.* 2015). Moreover, up-regulation of genes related to phospholipase C, Type VI secretion system and beta-lactamase by *Collimonas pratensis* as well as *Dyella sp*. in response to fungal presence further suggests that those bacteria

acquired nutrients from living hyphae by disrupting the fungal membrane and defense system (Song *et al.* 2015b; Journet & Cascales 2016). Interestingly, mycophageous or antifungal activity of *Dyella sp.* was not observed by direct-interaction assay with *F. culmorum*. However, it can be proposed that due to interaction with the bacteria in fungal presence the mycophageous lifestyle of *Dyella sp.* was stimulated, similarly as it was shown for the induction of antifungal activity by interactions of non-antagonistic bacteria (Garbeva & De Boer 2009).

Also on fungal side, a mycophagous livestyle by the bacteria was indicated. For example, the fungal biomass significantly decreased in bacterial presence and genes such as *hex-1* and *sed1p* that are related to repair systems in response to cell damage and confer resistance against lytic enzyme activity (Shimoi *et al.* 1998; Tenney *et al.* 2000) were expressed.

Genes encoding for properties involved in biofilm formation and twitching motility including Type II and Type IV secretion system (Burdman *et al.* 2011; Melville & Craig 2013; Johnson *et al.* 2014) as well as genes encoding for properties associated to adhesion to biotic surfaces such as Type I and IVb secretion system (Schilling et al. 2001; Bernard *et al.* 2009) were up-regulated in *Collimonas pratensis* and *Dyella sp*. in the presence of *F. culmorum*. Hence, those bacteria might have colonized and moved along the fungal hyphae to absorb nutrients that leaked out from the disrupted hyphal membrane. This is in line with a previous study showing the ability of *Dyella japonica* to migrate along hyphae of the fungus *Lyophyllum sp.* in soil microcosms Warmink and van Elsas (2009). The formation of biofilm by *Collimonas pratensis* and *Dyella sp*. at the hyphae of *F. culmorum* might also serve as protection to keep the acquired nutrients of the fungus for themselves and to prevent further leakage into soil.

Leaking out nutrients such as trehalose, histidine, and carnitine potentially produced by *F. culmorum* (Table S6.14) might serve as carbon and nitrogen source for the bacteria (Wargo & Hogan 2009; Stopnisek *et al.* 2016). Genes related to transport systems and degradation of those compounds were up-regulated by all bacteria, except *Paenibacillus sp*., in fungal presence. For the assimilation of nitrogen, bacteria evolved two major routes, (1) the glutamine synthetase and glutamate synthase cyclic mechanism or (2) nitrogen assimilation via the activity of glutamate dehydrogenase (Harper *et al.* 2010). Latter is induced in nitrogen excess. Interestingly, genes encoding for glutamate dehydrogenase were up-regulated by the bacteria such as *Burkholderia sp.*, *Collimonas pratensis*, and *Janthinobacterium sp.* in the presence of *F. culmorum* indicating that additional nitrogen was provided by the fungal hyphae. In contrast, the up-regulation of bacterial genes related to uptake of alternative phosphate sources such as glycerol-3-phosphate (Stopnisek *et al.*

2016) and genes related to phosphate starvation stress suggests that available phosphate was limited in fungal presence.

Generally, the presence of *F. culmorum* caused osmotic stress for the bacteria, indicated by the up-regulation of genes encoding for biosynthesis and transport systems of osmoregulatory solutes such as glycine betaine, proline or ectoine (Wood 2015). The occurrence of osmotic stress was also reported by Stopnisek *et al.* (2016) for the interaction of *Burkholderia* strains with various fungi. While an association of *Burkholderia* to fungi was reported by several studies (Warmink *et al.* 2009; Uroz *et al.* 2012; Nazir *et al.* 2013; Stopnisek *et al.* 2016), under the conditions used in our study, it seemed that the *Burkholderia* strain here was not able to colonize hyphae of *F. culmorum*. Genes involved in biofilm formation and defense mechanisms were down-regulated and the abundance was significantly decreased in in fungal presence. Moreover, the up-regulation of phospholipase *YtpA* producing the antifungal compound bacilysocin (Tamehiro *et al.* 2002) suggests that the interaction between *Burkholderia sp.* and *F. culmorum* was rather antagonistic. This is also indicated by a direct interaction assay performed earlier (Schulz-Bohm *et al.* 2017b).

The genus *Pseudomonas* is well known for its antagonistic activity against competing soil fungi (Weller 2007). They were often proposed as promising biocontrol agent against phytopathogenic fungi (Santoyo *et al.* 2012). However, the biocontrol activity of many pseudomonades under lab-condition was mostly not confirmed under field conditions (Hol *et al.* 2013). Also for *Pseudomonas sp.* used in this study the inhibition of *F. culmorum* observed by direct interaction assays (Schulz-Bohm *et al.* 2017b) stands in contrast to the decrease in abundance when incubated with other bacteria in the presence of *F. culmorum* in soil. Interspecific competitive interactions similar as occurred in this study in fungal presence may induce strong stress responses (i.e. osmotic and oxidative stress) at the expense of cell proliferation and biofilm formation, secondary metabolite production (i.e. terpenes, bioactive pigments, and antimicrobial nonribosomal peptides) as well as maintaining defense mechanisms (i.e. multidrug efflux systems and aromatic compound degradation). Moreover, genes related to antifungal features such as chitinase, proteases and the biosynthesis of antifungal compounds such as bacilysocin and pyrrolnitrin (Kirner *et al.* 1998; Tamehiro *et al.* 2002; De Boer *et al.* 2015) were down-regulated in *Pseudomonas sp.* in fungal presence.

Similar to *Pseudomonas sp.*, many genes including those related to secondary metabolite production and motility were strongly down-regulated in *Paenibacillus sp.* in the presence of *F. culmorum*. However, genes involved in spore formation were up-regulated indicating that the gram-positive strain switched to a dormant stage to overcome stress conditions.

In case of *Janthinobacterium sp*., genes encoding for methyl-accepting chemotaxis proteins and flagellar motor were up-regulated in fungal presence. Methyl-accepting chemotaxis proteins enable response to changing concentration of attractants as well as repellents by altering swimming behavior (Wadhams & Armitage 2004). Hence, it is plausible that *Janthinobacterium sp*. might have escaped from excreted fungal or bacterial antibiotics by simply moving away to explore new resources. A stimulation of swarming behavior of bacteria by competitive interactions was previously shown (De Boer *et al.* 2007; Garbeva & De Boer 2009; Garbeva *et al.* 2011b). In addition to motility, genes encoding for antitoxins were up-regulated in *Janthinobacterium sp.* in the presence of *F. culmorum*. The activity of those compounds potentially served as additional defense mechanism (Guglielmini & Van Melderen 2011).

In conclusion, by performing transcriptomics and volatolomics we could link the differential performance of bacteria in fungal presence to specific mechanisms. The best strategy to compete successfully in the presence of fungi points towards mycophagy in combination with the activation of secondary metabolite production and the induction of defense systems (Figure 6.6). This is in line with previous studies (Ballhausen & De Boer 2016) suggesting that mycophageous bacteria may be in generally successful competitors in fungal presence. In addition, stimulation of motility for exploitation of new resources and spore formation for the survival of stress conditions can be alternative strategies of bacteria in fungal presence (Figure 6.6). Generally, our study revealed that interspecific interactions played a significant role in the induction or inhibition of competitive traits including secondary metabolite production of bacteria. Therefore, studying interactions in synthetic microbial communities in combination with omics can give new insights for strategies to find effective biocontrol agents or novel secondary metabolites.

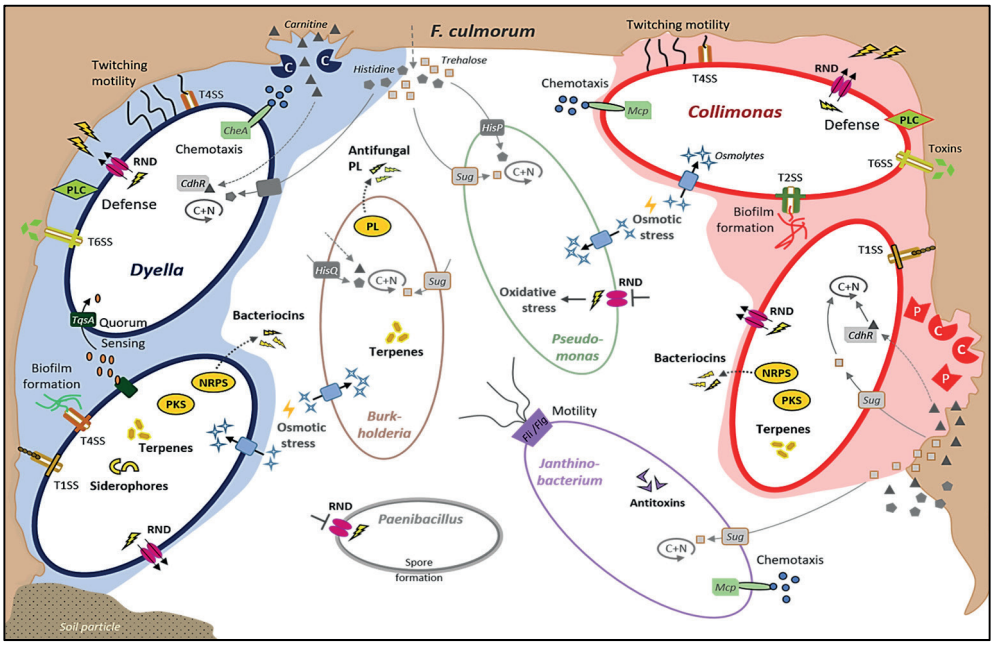

Figure 6.6. Schematic overview of important transcriptional changes of features explaining the different bacterial competitive strength in presence of the fungus *F. culmorum* in soil. The different shape size for each bacterium represent differences in cell abundance. Dotted arrows indicate potential pathways. Abbreviations: C, chitinase; GDH, glutamate dehydrogenase; NRPS, nonribosomal peptide synthase; P, Protease; PKS, polyketide synthase; PL, phospholipase or phospholipid; PLC, phospholipase C; RND, resistance nodulation cell division efflux system.

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Author contributions

KSB and PG contributed to conception and experiments designed. KSB performed all experiments, analysed and interpreted the data as well as created figures and tables. TJ conducted genome sequencing and assembly of two bacterial strains. FG gave bioinformatics support for genome annotation and the analysis of the RNA-Seq data. KSB drafted the manuscript with contributions from all co-authors.

Supplementary Material

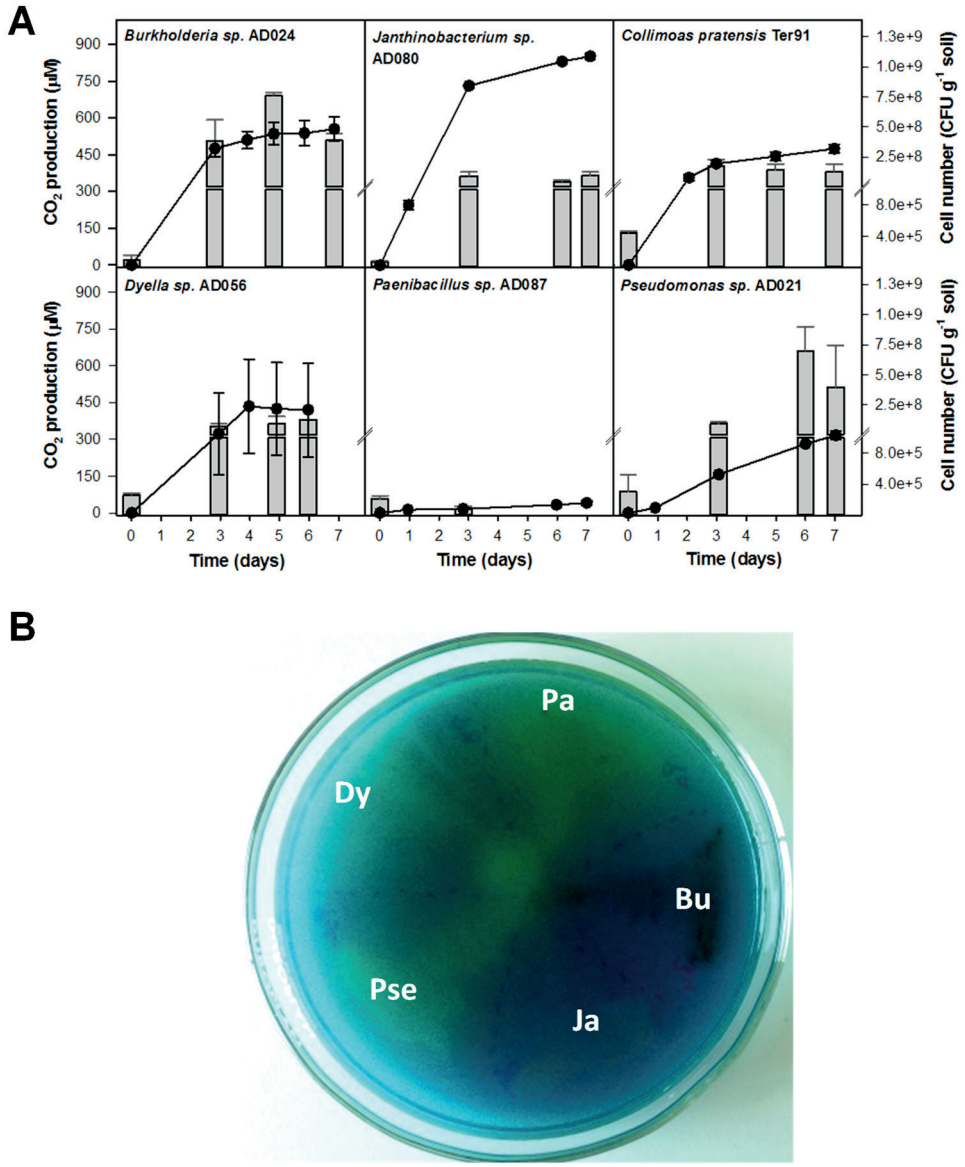

Figure S6.1. Growth and consumption of artificial root exudates in soil (A) and siderophore production (B) of bacteria isolated from the rhizosphere of *Carex arenaria* (see Table S6.1). **Panel A.** The CO_2-production profile (described by black lines including standard deviation) was similar for all bacteria, except *Paenibacillus sp.* A maximum was reached after 6 days of incubation indicating that supplied nutrients were fully consumed. The maximal cell number of the bacteria (illustrated by grey bars including standard deviation) was reached after 3-6 days of incubation. **Panel B.** Siderophore production is indicated by a yellow color stain of CAS medium as seen for *Paenibacillus sp.* AD87 (Pa) and *Pseudomonas sp.* AD21 (Pse).

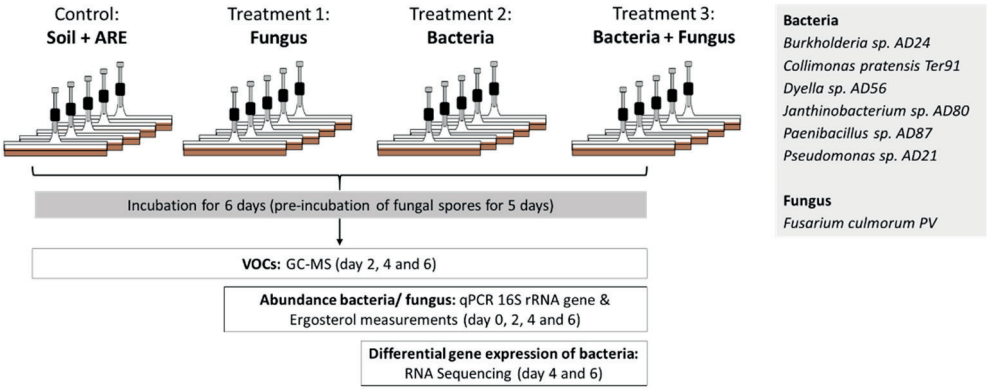

Figure S6.2. Experimental set-up to test the effect of fungal presence on the competitive strength of bacteria in rhizospheric soil microcosms. The microcosm experiment was performed in five replicates per treatment and the controls. Samples for the analysis of volatile organic compounds (VOCs), bacterial and fungal abundance as well differential gene expression of the bacteria were taken regularly within the incubation time of six days.

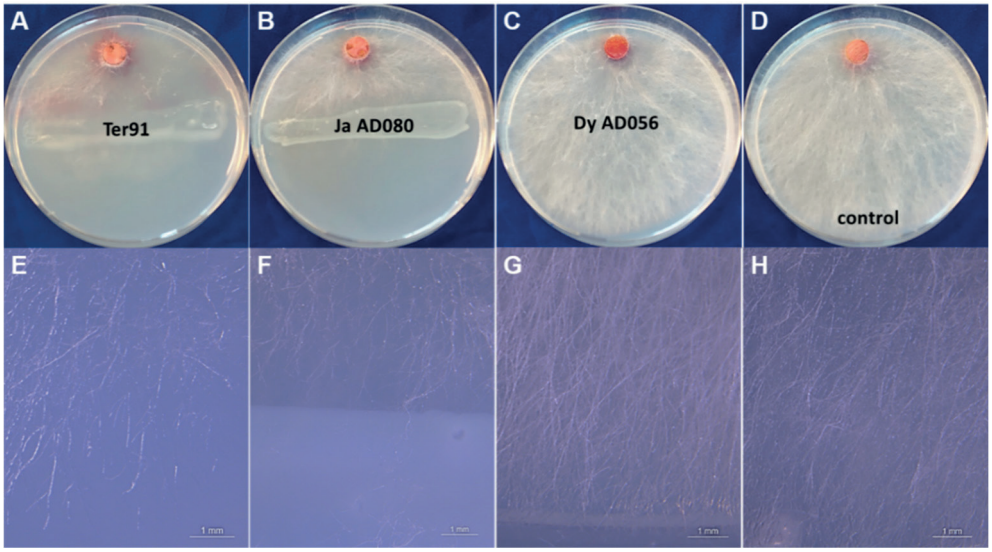

Figure S6.3. Direct interaction assay of F. culmorum PV with the bacteria Collimonas pratensis Ter91 (A and E), Dyella sp. AD56 (C and G), and Janthinobacterium sp. AD80 (B and F) on water-agar supplied with artificial root exudates. Bar in microscopic pictures of fungal hyphae (E-H) represent 1mm.

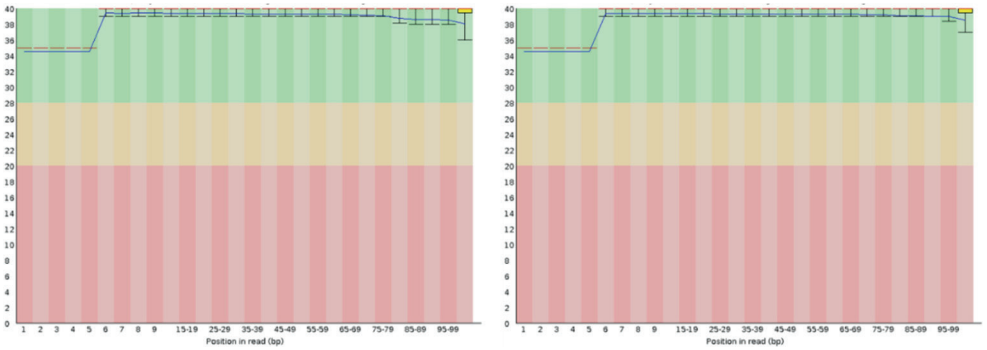

Figure S6.4. Quality score plots across all bases exemplary shown for two samples obtained from the treatments of bacteria in fungal absence (B) or presence (BF). The bars displayed in each plot are all located in the area marked in green demonstrating a high quality of the RNA sequencing reads.

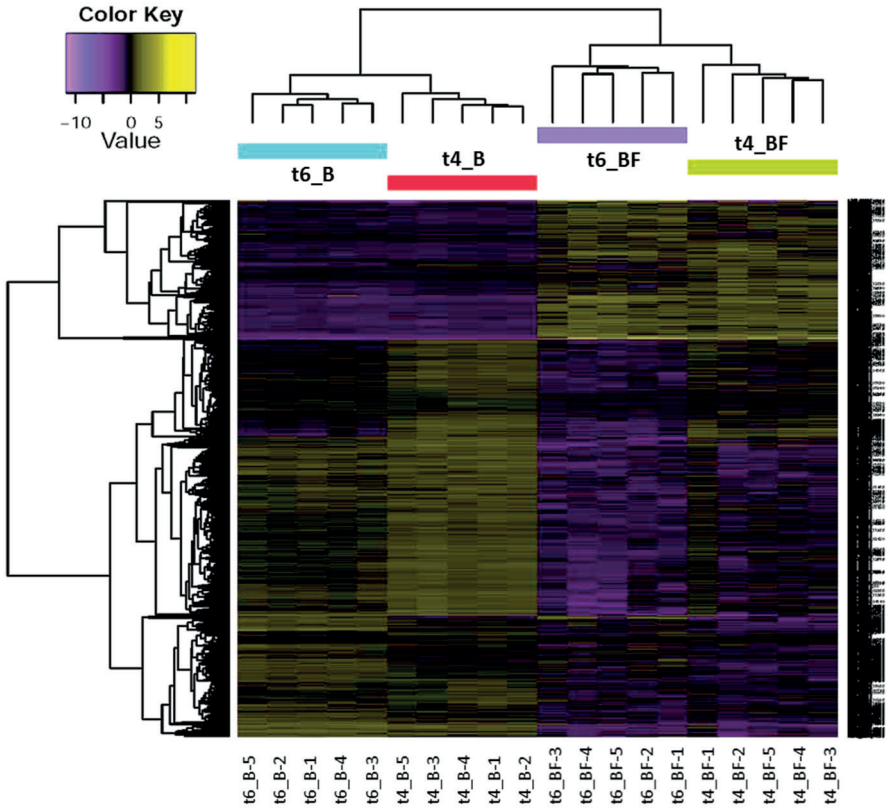

Figure S6.5. Heat-map of differentially expressed genes of bacteria in fungal absence (B) or presence (BF) after four days (t4) or six days (t6) of incubation. Data shown in the heat-map is obtained by analysis with EdgeR and DESeq2.

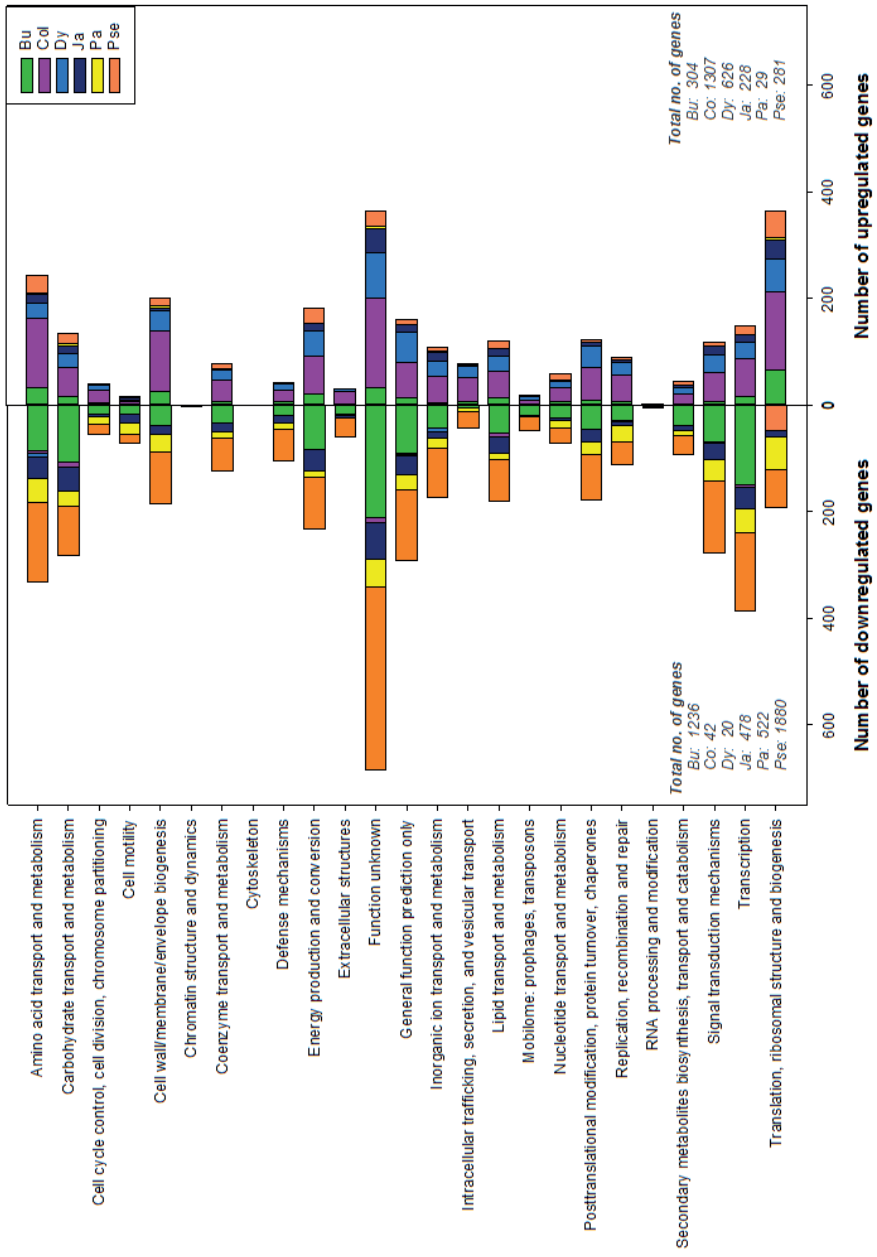

Figure S6.6. Differentially expressed genes assigned to various orthologous gene categories of bacteria in fungal presence. A bacterial synthetic community consistent of *Burkholderia sp.* AD24 (Bu), *Collimonas pratensis* Ter91 (Co), *Dyella sp.* AD56 (Dy), *Janthinobacterium sp*. AD80 (Ja), *Paenibacillus sp.* AD87 (Pa), and *Pseudomonas sp.* AD21 (Pse) was incubated in presence or absence of the fungus *F. culmorum* PV in rhizospheric soil for six days. Bacterial genes were account as up- or downregulated in the presence of the fungus with a p-value < 0.025 and a FDR value < 0.05. Total number of up- or downregulated genes per bacteria are listed at the bottom of the figure. Data were obtained by EdgeR and DESeq2 analaysis.

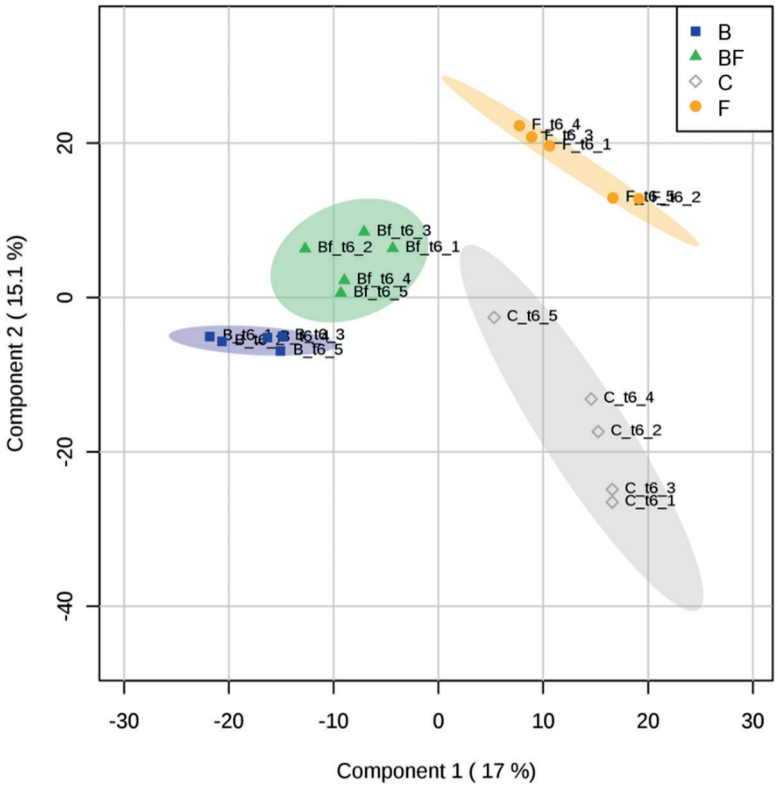

Figure S6.7. PLS-DA score plot of produced volatiles by *F. culmorum* PV (F) or the bacterial synthetic community in fungal presence (BF) or absence (B) in rhizospheric soil after 6 days of incubation. Control samples (C) consisting of soil and artificial root exudates were included in the analysis.

Table S6.1. Bacterial and fungal strains used in this study. All strains were isolated from the rhizosphere of *Carex arenaria* or *Ammophila arenaria* growing on sandy dune soils in the Netherlands. Bacteria were selected based on various antimicrobial traits such as siderophore production or antimicrobial activity as monoculture.

Bacteria and Fungus (Accesion number)	Origin	Phylum or Division *(family)*	Sidero-phore produc-tion	Antimicrobial activity
Burkholderia sp. AD24 (PRJNA320371)	Dune grassland near Ouddorp, Zeeland *(De Ridder-Duine et al. 2005)*	Beta-Proteobacteria *(Burkholderiaceae)*	No[1]	Antibacterial activity (direct interaction assay[3])
Collimonas pratensis Ter91 (CP013234)	Inner coastal dune soil in Terschelling *(De Boer et al. 2004)*	Beta-Proteobacteria *(Oxalobacteraceae)*	Yes[2]	Antifungal activity (mycophageous[2]; antifungal VOCs[4])
Dyella sp. AD56 (NRDP00000000)	Drift sand near Loon op Zand, Brabant *(De Ridder-Duine et al. 2005)*	Gamma-Proteobacteria (*Xantho-monadaceae*)	No[1]	Antibacterial activity (direct interaction assay[6])/ Antifungal activity (Antifungal VOCs[5])
Janthinobacterium sp. AD80 (NRDQ00000000)	Coastal outer dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	Beta-Proteobacteria *(Oxalobacteraceae)*	No[1]	Antibacterial activity (direct interaction assay[6])/ Antifungal activity (Antifungal VOCs[5])
Paenibacillus sp. AD87 (LXQN00000000)	Pine plantation near Loon op Zand, Brabant *(De Ridder-Duine et al. 2005)*	Firmicutes (Paenibacillaceae)	Yes[1]	Antibacterial activity (direct interaction assay[3, 6])
Pseudomonas sp. AD21 (NQYG00000000)	Coastal inner dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	Gamma-Proteobacteria *(Pseudo-monadaceae)*	Yes[1]	Antibacterial activity (direct interaction assay[6])
Fusarium culmorum PV	Dutch Coastal foredunes (*De Rooij-van der Goes et al. 1995*)	Ascomycota (*Nectriaceae*)	-	-

[1] see Figure S6.1 B
[2] Song *et al.* 2015b
[3] Tyc *et al.* 2017a
[4] Garbeva *et al.* 2014b
[5] Tyc *et al.* 2015
[6] Tyc *et al.* 2014

Table S6.2. Genome assembly properties of the bacteria *Dyella sp.* AD56, *Janthinobacterium sp.* AD80 and *Pseudomonas sp.* AD21.

Properties	*Dyella sp.* AD56	*Janthinobacterium sp.* AD80	*Pseudomonas sp.* AD21
Total bases	5,246,716	6,355,631	6,391,881
Number of contigs	55	394	231
CDS	4,643	5,682	5,632
genes	4722	5,815	5,781
misc_feature RNA	1	1	9
ncRNA	25	23	90
Regulatory RNA	9	7	6
rRNA	4	12	4
tRNA	49	96	46
tmRNA	1	1	1
Signal peptides	728	798	-

Table S6.3. Summary of number of reads obtained by RNA-Seq. Samples were collected from microcosm experiments of bacteria incubated for 4 days (t4) or 6 days (t6) in fungal absence (B) or presence (BF) in soil. Reads were filtered by using Fastq MCF (Aronesty 2011) and mapped against reference genomes using Bowtie 2 (Langmead & Salzberg 2012).

Sample	Raw	Filtered	Mapped against draft genomes
t4_B-1	23830403	23503925	15271877
t4_B-2	23817310	23476190	15345062
t4_B-3	24534759	24490780	16439103
t4_B-4	23545508	23436471	15453182
t4_B-5	24739481	24626354	16184071
t4_BF-1	24815072	25206369	1945426
t4_BF-2	24405594	24856005	1881344
t4_BF-3	24964819	25519597	1607606
t4_BF-4	23935569	24271049	2053079
t4_BF-5	23704850	24155915	2081615
t6_B-1	24086090	23433504	14907427
t6_B-2	23942077	23577109	15539586
t6_B-3	24685958	24630016	16082005
t6_B-4	23954375	23542816	15583664
t6_B-5	24547024	24016912	15697010
t6_BF-1	26598901	27181836	1589541
t6_BF-2	24241575	24613604	1528053
t6_BF-3	24883688	25221332	1793229
t6_BF-4	23899789	24379009	1436960
t6_BF-5	24289043	24784717	1803630

Table S6.4. Differentially expressed genes related to various stress responses of bacteria in presence of *F. culmorum* in rhizospheric soil after four days of incubation. Upregulated and downregulated genes in fungal presence are indicated by log2FC > 0 and log2FC < 0, respectively.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Osmotic stress		
AD21_09560	Choline-sulfatase[A]	6.99	5.91E-31
AD21_09565	Glycine betaine/carnitine transport binding protein GbuC[B]	5.31	2.97E-29
AD21_21255	NAD/NADP-dependent betaine aldehyde dehydrogenase[C]	4.84	9.15E-84
AD21_21250	HTH-type transcriptional regulator BetL[C]	4.65	3.12E-92
AD21_21245	High-affinity choline transport protein[D]	4.33	6.78E-71
AD21_21260	Oxygen-dependent choline dehydrogenase[D]	3.67	1.85E-39
AD21_07610	Glycine betaine-binding protein OpuAC[E]	2.33	5.21E-34
AD21_21230	Glycine betaine/carnitine transport binding protein GbuC[B]	1.70	1.54E-18
AD21_09810	Glycine betaine-binding periplasmic protein[E]	1.03	0.007
AD21_20025	Proline-specific permease ProY	0.80	0.012
AD21_04600	High-affinity choline transport protein[D]	0.80	0.028
AD21_21235	Glycine betaine transport system permease protein OpuAB[E]	0.74	4.14E-05
AD21_15340	Osmotically-inducible protein Y	-1.72	2.88E-09
AD21_22360	Proline/betaine transporter[F]	-1.64	1.15E-07
bAD24_chr2_13435	HTH-type transcriptional regulator BetL[C]	5.21	3.69E-16
bAD24_chr2_13565	Glycine betaine/L-proline transport ATP-binding protein ProV[G]	3.63	9.13E-41
bAD24_chr2_13440	NAD/NADP-dependent betaine aldehyde dehydrogenase[C]	3.20	2.45E-29
bAD24_chr2_13560	Glycine betaine/carnitine transport permease protein GbuB[B]	2.81	4.55E-07
bAD24_chr2_13375	Glycine betaine-binding protein OpuAC[E]	1.77	0.002
bAD24_chr2_13445	Oxygen-dependent choline dehydrogenase[D]	1.31	0.006
bAD24_chr2_13550	Glycine betaine/carnitine transport binding protein GbuC[B]	0.86	0.027
bAD24_chr2_01455	NAD/NADP-dependent betaine aldehyde dehydrogenase[C]	-2.18	5.79E-14
bAD24_chr1_10440	Ectoine dioxygenase[H]	-1.10	5.64E-05
bAD24_chr2_11535	Osmotically-inducible protein Y[F]	-0.93	1.30E-06
bAD24_chr2_13615	Osmotically-inducible protein Y[F]	-0.92	0.003
AD56_12945	Proline/betaine transporter[F]	5.51	1.68E-63
AD56_04160	Transcriptional regulatory protein OmpR[I]	3.98	0.011
AD56_05225	Hypothetical protein (Nucleoid-associated protein YgaU)[J]	1.98	0.016
AD56_09955	RNA polymerase-associated protein RapA[K]	1.86	2.05E-05
AD56_06100	RNA polymerase-associated protein RapA[K]	1.19	0.034
AD80_04775	Hypothetical protein (Ectoine hydroxylase-related dioxygenase)[H]	2.18	1.19E-08
Ter91_01395	Ectoine dioxygenase[H]	2.93	0.001
Ter91_14550	Proline/betaine transporter[F]	1.85	0.019
Ter91_02625	Ectoine dioxygenase[H]	1.32	0.011
Ter91_04870	Proline/betaine transporter[F]	1.32	0.001
Ter91_16840	Proline/betaine transporter[G]	1.11	2.37E-08

Table S6.4 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Oxidative stress		
AD21_25110	8-oxoguanine deaminase[L]	0.68	0.024
AD21_12955	Superoxide dismutase [Mn/Fe]	-1.65	0.003
AD21_19895	Multicopper oxidase[M]	-1.52	1.98E-07
bAD24_chr1_19875	8-oxoguanine deaminase[L]	-0.96	0.013
AD56_13330	Free methionine-R-sulfoxide reductase[N]	2.52	4.79E-07
AD56_03145	Superoxide dismutase [Fe]	1.38	1.09E-08
AD56_20515	Metalloregulation DNA-binding stress protein[O]	0.96	0.005
AD80_07870	Superoxide dismutase [Cu-Zn] 1	-1.12	2.87E-04
AD87_11175	8-oxoguanine deaminase[L]	-6.40	0.003
AD87_30160	Superoxide dismutase [Mn/Fe]	-1.72	0.006
AD87_30210	Superoxide dismutase [Mn]	-1.15	1.43E-04
	Phosphate limitation/ Starvation stress		
AD21_02380	Phosphate starvation-inducible protein PsiF	-1.56	5.07E-18
AD21_22875	GTP pyrophosphokinase[P]	-1.49	3.47E-19
AD21_16090	Stringent starvation protein B	-1.43	6.89E-09
AD21_03450	Phosphate starvation-inducible protein PhoH	-1.36	3.54E-12
AD21_06710	DNA protection during starvation protein 2	-1.31	1.49E-04
AD21_16085	Stringent starvation protein A	-1.19	1.57E-09
AD21_15060	Phosphate starvation-inducible protein PhoH	-1.09	1.16E-10
AD21_15280	ncRNA CrcZ[Q]	-1.06	5.14E-13
bAD24_chr1_08465	DNA protection during starvation protein 2	1.39	1.01E-05
AD56_00150	Carbon starvation protein A	3.11	1.35E-09
AD56_03310	Phosphate starvation-inducible protein PsiF	2.75	4.89E-16
AD56_03110	hypothetical protein (Stringent starvation protein B)	2.40	2.00E-04
AD56_03065	Phosphate starvation-inducible protein PhoH	2.19	7.70E-04
AD56_18335	Outer membrane protein SLP (Starvation-inducible outer membrane lipoprotein)	2.16	4.85E-05
AD56_00560	GTP pyrophosphokinase[P]	1.57	0.016
AD56_07715	Alkaline phosphatase synthesis transcriptional regulatory protein SphR[R]	1.43	1.34E-04
AD80_05665	Phosphate regulon transcriptional regulatory protein PhoB	-2.29	0.001
AD87_01705	GTP pyrophosphokinase[P]	-1.40	1.56E-04

Table S6.4 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Oxidative stress		
Ter91_16755	HTH-type transcriptional repressor RspR[S]	3.97	4.69E-15
Ter91_15175	Phosphate regulon sensor protein PhoR	2.19	3.48E-06
Ter91_04375	Phosphate regulon transcriptional regulatory protein PhoB	1.80	2.91E-21
Ter91_03700	Stringent starvation protein A	1.79	5.18E-16
Ter91_03705	Stringent starvation protein B	1.78	2.36E-06
Ter91_20730	Phosphate starvation-inducible protein PhoH	1.61	6.71E-10
Ter91_15180	Phosphate regulon transcriptional regulatory protein PhoB	1.40	0.006
Ter91_15035	Phosphate starvation-inducible protein PhoH	0.94	1.91E-06

[1] AD21, *Pseudomonas sp.*; bAD24, *Burkholderia sp.*; AD56, *Dyella sp.*, AD80, *Janthinobacterium sp.*; AD87, *Paenibacillus sp.* AD87; Ter91, *Collimonas pratensis*.

[2] COG description is indicated and used for further identification of the function of the gene classified as hypothetical proteins..

[3] Log2 Fold change.

[4] False discovery rate.

[A] Involved in osmotic stress resistance (Boscari et al. 2002; Østerås et al. 1998).

[B] Part of the ABC transporter complex GbuABC involved in osmoprotection by glycine betaine uptake (Angelidis & Smith 2003).

[C] Involved in the biosynthesis of the osmoprotectant glycine betaine (Falkenberg & Strom 1990; Rkenes et al. 1996).

[D] Involved in osmotic stress resistance. Choline dehydrogenase catalyses the conversion of choline into the intermediate glycine betaine aldehyde, as part of a two-step oxidative reaction leading to the formation of osmoprotectant betaine (Boscari et al. 2002).

[E] Member of the ABC transporter superfamily that function as a high-affinity uptake system for the osmoprotectant glycine betaine (Kempf et al. 1997).

[F] Proton symporter that senses osmotic shifts and responds by importing osmolytes such as proline or glycine betaine. It is both an osmosensor and an osmoregulator which is available to participate early in the bacterial osmoregulatory response (Racher et al. 1999).

[G] Part of the ProU ABC transporter complex involved in glycine betaine and proline betaine uptake (Gul & Poolman 2013).

[H] Ectoine dioxygenase EctD is involved in the biosynthesis of ectoine ((S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) which is a highly soluble organic osmolyte (Prabhu et al. 2004).

[I] EnvZ/OmpR is a two-component regulatory system involved in osmoregulation (Cai & Inouye 2002).

[J] Involved in osmotic stress resistance (Fitzgerald *et al.* 2015).

[K] Transcription regulator that activates transcription by stimulating RNA polymerase recycling in case of stress conditions such as high salt concentrations (Sukhodolets et al. 2001).

[L] Specifically deaminates 8-Oxoguanine (8-oxoG) to uric acid. 8-oxoG is formed via the oxidation of guanine within DNA by reactive oxygen species (Hall et al. 2010).

[M] Potentially involved in oxidative stress response (Sitthisak et al. 2005).

[N] Met-(R)-O represent a signaling molecule in response to oxidative stress (Lin et al. 2007).

[O] Protection DNA against oxidative stress (Martinez & Kolter 1997).

[P] ppGpp (guanosine 3'-diphosphate 5-' diphosphate) is a mediator of the stringent response that coordinates a variety of cellular activities in response to changes in nutritional abundance (Zhang et al. 2016).

[Q] CrcZ is a small RNA found in Pseudomonas bacteria, which acts as a global regulator of carbon catabolite repression (Sonnleitner et al. 2009).

[R] Response regulator involved in inducible production of alkaline phosphatase in response to phosphate limitation (Nagaya et al. 1994).

[S] Repressor of the rspAB operon that is involved in the starvation response (Hancock et al. 2008).

Table S6.5. Differentially expressed genes of bacteria related to chemotaxis in presence of *F. culmorum* in rhizospheric soil after four days of incubation. Upregulated and downregulated genes in fungal presence are indicated by log2FC > 0 and log2FC < 0, respectively.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
AD21_15950	Methyl-accepting chemotaxis protein PctB	1.50	1.06E-15
AD21_28470	Methyl-accepting chemotaxis protein McpS	0.91	1.64E-07
AD21_17400	Methyl-accepting chemotaxis protein McpS	-2.17	2.77E-16
AD21_23540	Methyl-accepting chemotaxis protein McpS	-2.10	1.52E-14
AD21_10115	Methyl-accepting chemotaxis protein PctB	-1.67	9.83E-10
AD21_01565	Chemotaxis protein CheY	-1.64	1.29E-11
AD21_18895	Chemotaxis protein CheY	-1.63	5.55E-11
AD21_24125	Chemotaxis protein CheA	-1.62	2.11E-12
AD21_00275	Chemotaxis protein CheV	-1.54	1.21E-14
AD21_26535	Methyl-accepting chemotaxis protein McpS	-1.36	1.97E-10
AD21_11360	Methyl-accepting chemotaxis protein PctB	-1.29	3.73E-12
AD21_24705	Chemotaxis response regulator protein-glutamate methylesterase	-1.25	1.10E-06
AD21_11350	Methyl-accepting chemotaxis protein PctC	-1.25	1.10E-07
AD21_23715	Methyl-accepting chemotaxis protein PctA	-1.22	1.54E-08
AD21_13470	Chemotaxis response regulator protein-glutamate methylesterase	-1.17	0.002
AD21_16280	Chemotaxis protein CheV	-1.13	1.04E-12
AD21_06620	Methyl-accepting chemotaxis protein PctA	-1.10	5.33E-11
AD21_24115	Chemotaxis protein CheW	-1.07	2.53E-05
AD21_01520	Chemotaxis protein methyltransferase Cher2	-1.07	3.12E-08
AD21_23305	Methyl-accepting chemotaxis protein PctC	-1.06	1.04E-06
AD21_26030	Methyl-accepting chemotaxis protein PctB	-1.04	1.71E-12
AD21_07860	Chemotaxis protein CheA	-1.03	2.53E-09
AD21_07855	Chemotaxis response regulator protein-glutamate methylesterase of group 1 operon	-0.97	1.22E-04
AD21_07475	Methyl-accepting chemotaxis protein McpS	-0.94	2.91E-05
AD21_12195	Methyl-accepting chemotaxis protein McpS	-0.91	1.41E-10
bAD24_chr1_17570	Methyl-accepting chemotaxis protein II	0.77	0.005
bAD24_chr1_11800	Chemotaxis protein methyltransferase	-6.99	8.10E-05
bAD24_chr1_02255	hypothetical protein (Methyl-accepting chemotaxis protein)	-2.52	4.40E-13
bAD24_chr1_11790	Chemotaxis protein CheW	-1.97	0.005
bAD24_chr1_10990	Hypothetical protein (Chemotaxis signal transduction protein)	-1.75	0.010
bAD24_chr1_11785	Chemotaxis protein CheA	-1.48	4.27E-12
AD56_07625	Chemotaxis protein CheA	0.81	0.044

Table S6.5 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR
AD80_13255	Methyl-accepting chemotaxis protein I	2.76	1.68E-04
AD80_08455	Methyl-accepting chemotaxis protein II	1.22	3.22E-05
AD80_02810	Methyl-accepting chemotaxis protein II	1.19	1.44E-05
AD80_14030	Methyl-accepting chemotaxis protein III	1.06	8.65E-07
AD80_09020	Methyl-accepting chemotaxis protein II	0.93	0.034
AD80_08420	Methyl-accepting chemotaxis protein II	0.76	0.030
AD80_19530	Chemotaxis protein CheY	-1.16	6.17E-05
AD80_20705	Chemotaxis protein LafU	-1.04	5.57E-05
AD80_09470	Chemotaxis response regulator protein-glutamate methylesterase	-0.94	3.47E-04
AD87_27145	Methyl-accepting chemotaxis protein McpB	-7.06	6.90E-05
AD87_30925	Methyl-accepting chemotaxis protein McpA	-6.86	0.002
AD87_00740	CheY-P phosphatase (Chemotaxis protein CheY)	-3.49	-11.2182
AD87_31770	Methyl-accepting chemotaxis protein McpB	-3.24	0.001
AD87_16080	Methyl-accepting chemotaxis protein McpB	-3.18	0.008
AD87_03075	Methyl-accepting chemotaxis protein McpB	-3.10	5.90E-04
AD87_06265	Chemotaxis protein methyltransferase Cher2	-2.98	7.29E-07
AD87_15580	Chemotaxis protein CheY	-2.86	2.42E-04
AD87_02770	Methyl-accepting chemotaxis protein McpB	-2.83	1.93E-06
AD87_15535	Chemotaxis response regulator protein-glutamate methylesterase	-2.76	2.88E-04
AD87_02695	Methyl-accepting chemotaxis protein McpB	-2.26	1.37E-04
AD87_17665	Hypothetical protein (Methylase of chemotaxis methyl-accepting proteins)	-2.13	3.65E-04
AD87_01875	Methyl-accepting chemotaxis protein McpC	-2.09	5.90E-05
AD87_21065	Methyl-accepting chemotaxis protein McpA	-2.03	0.016
AD87_15530	Chemotaxis protein CheA	-1.85	0.005
AD87_27755	Putative sensory transducer protein YfmS (Methylase of chemotaxis methyl-accepting proteins)	-1.54	0.006
Ter91_14410	Methyl-accepting chemotaxis protein McpB	2.14	1.36E-19
Ter91_04385	Methyl-accepting chemotaxis protein PctA	1.64	5.58E-19
Ter91_04390	Chemotaxis protein CheA	1.53	1.21E-18
Ter91_04380	Hypothetical protein (Chemotaxis signal transduction protein)	1.47	1.45E-04

[1] AD21, *Pseudomonas sp.*; bAD24, *Burkholderia sp.*; AD56, *Dyella sp.*, AD80, *Janthinobacterium sp.*; AD87, *Paenibacillus sp.*; Ter91, *Collimonas pratensis*.
[2] COG description is indicated and used for further identification of the function of the gene classified as hypothetical proteins..
[3] Log2 Fold change.
[4] False discovery rate.

Table S6.6. Differentially expressed genes of bacteria related to cell motility in presence of *F. culmorum* in rhizospheric soil after four days of incubation. Up-regulated and down-regulated genes in fungal presence are indicated by Log2FC > 0 and Log2FC < 0, respectively.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
AD21_08185	Flagellar basal-body rod protein FlgF[A]	0.72	9.01E-04
AD21_14555	Hypothetical protein (Tfp pilus assembly protein FimV)[D]	-3.11	0.003
AD21_08360	Hypothetical protein (Tfp pilus assembly protein FimV)[D]	-2.20	5.72E-19
AD21_08735	Twitching mobility protein[D]	-1.87	1.56E-11
AD21_07845	Motility protein B (MotB)[A]	-1.85	1.49E-12
AD21_10685	RNA polymerase sigma factor RpoS[D]	-1.66	2.71E-15
AD21_07990	Flagellar FliJ protein[A]	-1.65	2.78E-05
AD21_07880	Flagellum site-determining protein YlxH (FleN/FlhG)[A]	-1.53	2.98E-11
AD21_24055	Twitching mobility protein[D]	-1.45	1.25E-06
AD21_08040	Flagellar protein FliS[A]	-1.32	1.35E-14
AD21_23730	Hypothetical protein (Tfp pilus assembly protein FimV)[D]	-1.28	4.54E-12
AD21_08055	Flagellin (FliC)[A]	-1.24	6.69E-14
AD21_08160	Flagellar hook-associated protein 1 (FlgK) [A]	-1.23	1.02E-16
AD21_23195	Motility protein A (MotA) [A]	-1.22	5.16E-13
AD21_05550	Hypothetical protein (Flagellar basal body-associated protein FliL)[A]	-1.19	1.24E-10
AD21_14710	Flagellar brake protein YcgR[B]	-1.18	9.56E-06
AD21_23200	Motility protein B (MotB)[A]	-1.17	9.30E-09
AD21_08045	Flagellar hook-associated protein 2 (FliD)[A]	-1.12	2.00E-09
AD21_08155	Flagellar hook-associated protein 3 (FlgL)[A]	-1.04	3.73E-05
AD21_01540	Hypothetical protein (Flagellar biosynthesis)	-0.93	1.75E-09
AD21_01500	Flagellar hook protein FlgE[A]	-0.91	3.99E-06
bAD24_chr2_02390	Hypothetical protein (Tfp pilus assembly protein FimV)[D]	-1.85	0.002
bAD24_chr1_07040	Hypothetical protein (Flagellar capping protein FliD)[A]	-1.67	6.07E-05
bAD24_chr1_07025	Flagellar hook-associated protein 2 (FliD)[A]	-1.62	3.60E-07
bAD24_chr2_01270	Motility protein A (MotA)[A]	-1.43	2.01E-04
bAD24_chr1_07030	A-type flagellin (FliC)[A]	-1.19	3.81E-04
bAD24_chr2_08485	Flagellar transcriptional regulator FlhD[A]	-1.12	1.16E-08
bAD24_chr2_08480	Flagellar transcriptional regulator FlhC[A]	-1.07	8.39E-06
bAD24_chr1_07035	Flagellar hook protein FlgE[A]	-0.99	1.43E-04
AD56_16395	Flagellar biosynthesis protein FlhF[A]	2.76	0.042
AD56_08360	Hypothetical protein (Tfp pilus assembly protein FimV)[D]	2.11	1.03E-05
AD56_07055	Twitching mobility protein[D]	1.88	8.06E-06
AD56_07555	Twitching mobility protein[D]	1.73	0.011
AD56_21910	Hypothetical protein (Flagellar basal body rod protein FlgG)[A]	1.66	4.10E-04
AD56_03125	Hypothetical protein (Tfp pilus assembly protein FimV)	1.63	2.20E-05
AD56_07720	Sensor protein QseC[C]	1.56	0.012
AD56_10065	Sensor protein QseB[C]	1.46	0.049

Table S6.6 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
AD80_13505	Flagellin (FliC)[A]	2.47	6.57E-04
AD80_13495	Flagellin (FliC)[A]	2.32	5.93E-16
AD80_13500	Flagellar filament 33 kDa core protein	2.00	4.36E-14
AD80_13490	Flagellin (FliC)[A]	1.90	3.03E-12
AD80_13510	Flagellin (FliC)[A]	1.82	4.51E-05
AD80_20630	Flagellar hook-associated protein 3 (FlgL)[A]	1.05	0.002
AD80_20635	Flagellar hook-associated protein 1 (FlgK)[A]	0.96	0.003
AD80_13590	Flagellar protein FliS[A]	0.96	0.002
AD80_13585	Flagellar hook-associated protein 2 (FliD)[A]	0.85	0.002
AD80_26525	Flagellar hook-length control protein FliK[A]	-2.38	5.38E-04
AD80_22440	Peptidoglycan hydrolase FlgJ[A]	-1.88	1.54E-04
AD80_22430	Flagellar L-ring protein (FlgH)[A]	-1.86	0.002
AD80_21085	Flagellar basal body rod protein FlgB[A]	-1.51	0.002
AD80_25620	Flagellar hook-basal body complex protein FliE[A]	-1.43	0.004
AD80_25630	Flagellar motor switch protein FliG[A]	-1.16	9.55E-04
AD80_26515	Flagellar motor switch protein FliM[A]	-0.99	1.34E-04
AD80_28775	RNA polymerase sigma factor RpoS[D]	-0.88	3.23E-04
AD87_28265	Flagellar protein FliS[A]	-7.18	2.13E-05
AD87_15555	Flagellar biosynthetic protein FlhB[A]	-3.96	2.53E-09
AD87_06125	Flagellar brake protein YcgR[B]	-3.70	1.27E-04
AD87_15550	Flagellar biosynthesis protein FlhA[A]	-3.44	7.58E-11
AD87_15560	Flagellar biosynthetic protein FliR[A]	-3.37	2.91E-06
AD87_28235	Flagellar filament 30.7 kDa core protein	-3.13	1.95E-16
AD87_15620	Flagellar hook-length control protein FliK[A]	-3.11	2.82E-09
AD87_15615	Basal-body rod modification protein FlgD[A]	-3.09	1.04E-06
AD87_15645	Flagellar motor switch protein FliG[A]	-2.99	1.80E-04
AD87_27830	Swarming motility protein SwrC	-2.82	4.93E-07
AD87_28090	Flagellar basal-body rod protein FlgG[A]	-2.79	7.20E-07
AD87_28260	Flagellar hook-associated protein 2 (FlgK)[A]	-2.45	1.77E-11
AD87_15575	Hypothetical protein (Flagellar biogenesis protein FliO)[A]	-2.29	1.94E-07
AD87_28250	Flagellin (FliC)[A]	-2.27	2.08E-13
AD87_24655	Motility protein B (MotB)[A]	-2.27	2.78E-06
AD87_28230	Flagellar hook-associated protein 1 (FlgK)[A]	-2.26	1.87E-07
AD87_15545	Flagellar biosynthesis protein FlhF[A]	-2.25	3.96E-04
AD87_28100	Flagellar basal-body rod protein FlgG[A]	-2.21	1.93E-05
AD87_02260	Hypothetical protein (Flagellar motility protein MotE)[A]	-2.01	4.75E-06
AD87_15605	Flagellar basal-body rod protein FlgG[A]	-1.92	1.30E-04
AD87_15625	Hypothetical protein (Flagellar motility protein MotE)[A]	-1.85	0.004
AD87_15570	Flagellar biosynthetic protein FliP[A]	-1.44	0.010
AD87_15590	Flagellar motor switch protein FliM[A]	-1.39	0.006
AD87_15585	Flagellar motor switch protein FliN[A]	-1.38	0.001

Table S6.6 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
Ter91_14430	Gliding motility regulatory protein	1.61	4.10E-04
Ter91_04175	Twitching mobility protein[D]	1.00	2.18E-04
Ter91_17610	Twitching mobility protein[D]	0.63	0.023

[1] AD21, *Pseudomonas sp.*; bAD24, *Burkholderia sp.*; AD56, *Dyella sp.*, AD80, *Janthinobacterium sp.*; AD87, *Paenibacillus sp.*; Ter91, *Collimonas pratensis*.
[2] COG description is indicated and used for further identification of the function of the gene classified as hypothetical proteins..
[3] Log2 Fold change.
[4] False discovery rate.
[A] Part of flagellar motor (Morimoto & Minamino 2014).
[B] Controls Flagellar Motor Direction (Paul et al. 2010).
[C] Member of a two-component quorum sensing regulatory system QseB/QseC involved in the regulation of flagella and motility (Sperandio et al. 2002).
[D] Involved in twitching motility (Semmler *et al.* 2000).

Table S6.7. Differentially expressed genes of bacteria related to biofilm formation and attachment to abiotic or biotic surfaces in presence of *F. culmorum* in rhizospheric soil after four days of incubation. Up-regulated and down-regulated genes in fungal presence are indicated by Log2FC > 0 and Log2FC < 0, respectively.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
AD21_18905	Hypothetical protein (Flp pilus assembly protein TadG)[A]	-3.10	2.56E-17
AD21_07155	Adhesin Ata autotransporter	-2.89	1.50E-21
AD21_18855	Hypothetical protein (Flp pilus assembly protein TadB)[A]	-2.49	1.61E-12
AD21_18880	Hypothetical protein (Flp pilus assembly protein, pilin Flp)[B]	-2.39	5.00E-21
AD21_18885	Hypothetical protein (Flp pilus assembly protein, pilin Flp)[B]	-2.32	5.98E-16
AD21_18875	Hypothetical protein (Flp pilus assembly protein CpaB)[C]	-2.30	3.39E-19
AD21_17875	Outer membrane usher protein FimD[D]	-2.27	9.17E-10
AD21_15350	Type II secretion system protein E[E]	-2.12	1.08E-17
AD21_18825	Hypothetical protein (Autotransporter adhesion)	-2.10	1.76E-16
AD21_15475	Hypothetical protein (Tfp pilus assembly protein PilV)[F]	-2.07	1.94E-09
AD21_15480	Hypothetical protein (Tfp pilus assembly protein FimT)[F]	-1.96	4.19E-15
AD21_18870	Putative type II secretion system protein D[E]	-1.91	8.85E-14
AD21_18900	Hypothetical protein (Flp pilus assembly protein CpaA)[C]	-1.88	1.20E-04
AD21_18850	Hypothetical protein (Pilus assembly protein TadC)[A]	-1.86	2.37E-12
AD21_10330	Hypothetical protein (Tfp pilus assembly protein PilN)[F]	-1.82	0.004
AD21_18515	Biofilm dispersion protein BdlA	-1.79	4.97E-22
AD21_15470	Hypothetical protein (Tfp pilus assembly protein PilW)[F]	-1.73	7.79E-06
AD21_15345	Fimbrial protein[F]	-1.70	2.65E-15
AD21_18865	Hypothetical protein (Flp pilus assembly protein CpaE)[C]	-1.70	6.28E-14
AD21_15485	Hypothetical protein (Tfp pilus assembly protein FimT)[F]	-1.68	0.002
AD21_21485	Alginate lyase[G]	-1.57	3.99E-13
AD21_09045	Hypothetical protein (Tfp pilus assembly protein PilZ)[F]	-1.56	1.41E-08
AD21_25810	Hypothetical protein (Acetyltransferase involved in cellulose biosynthesis)	-1.51	1.57E-07

Table S6.7 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
AD21_18845	Hypothetical protein (Flp pilus assembly protein TadD)[A]	-1.48	2.91E-12
AD21_26170	Putative diguanylate cyclase AdrA[H] (Two-component response regulator, PleD family)	-1.46	1.04E-07
AD21_15360	Type 4 prepilin-like proteins leader peptide-processing enzyme[F]	-1.45	2.74E-04
AD21_04940	Poly-beta-1,6-N-acetyl-D-glucosamine export protein	-1.44	2.38E-10
AD21_09460	Alginate biosynthesis sensor protein KinB[G]	-1.41	1.37E-07
AD21_04925	Biofilm PGA synthesis protein PgaD	-1.40	2.81E-05
AD21_01850	Putative diguanylate cyclase AdrA[H]	-1.31	1.43E-05
AD21_25835	Poly-beta-1,6-N-acetyl-D-glucosamine synthase	-1.24	3.58E-05
AD21_15460	Type IV pilus biogenesis factor PilY1[F]	-1.20	9.83E-06
AD21_09465	Alginate biosynthesis transcriptional regulatory protein AlgB[G]	-1.17	3.83E-06
AD21_04930	Poly-beta-1,6-N-acetyl-D-glucosamine synthase	-1.15	5.08E-07
AD21_10325	Hypothetical protein (Tfp pilus assembly protein PilO)[F]	-1.09	0.004
AD21_00765	Alginate biosynthesis protein AlgA[G]	-1.04	4.72E-12
AD21_05660	Type II secretion system protein E[E]	-0.99	6.79E-04
AD21_13730	Inner membrane protein YhaI (Tfp pilus assembly protein, major pilin PilA)[F]	-0.90	0.001
AD21_25835	Poly-beta-1,6-N-acetyl-D-glucosamine synthase	-0.90	5.22E-07
bAD24_chr2_04350	Adhesin Ata autotransporter	-1.55	0.002
bAD24_chr1_03810	Hypothetical protein (Autotransporter adhesion)	-1.53	4.32E-18
bAD24_chr1_00330	Hypothetical protein (catalytic subunit of cellulose synthase and poly-beta-1,6-N-acetylglucosamine synthase)	-1.49	1.30E-06
bAD24_chr2_06815	Hypothetical protein (Autotransporter adhesion)	-1.41	1.19E-09
bAD24_chr2_12165	AI-2 transport protein TqsA[I]	-1.20	2.41E-05
bAD24_chr1_03815	Hypothetical protein (Autotransporter adhesion)	-1.13	4.74E-09
bAD24_chr2_09635	Hypothetical protein (Autotransporter adhesion)	-1.09	3.08E-09
bAD24_chr1_06175	Putative fimbrial chaperone YadV[J]	-1.07	1.07E-06
bAD24_chr1_03790	Hypothetical protein (Flp pilus assembly protein CpaB)[C]	-0.91	2.02E-07
AD56_06300	Thermolabile hemolysin[K]	3.67	0.011
AD56_15105	Hypothetical protein (Tfp pilus assembly protein FimT)[F]	3.58	7.20E-06
AD56_10755	Putative diguanylate cyclase YdaM[J]	3.22	7.19E-04
AD56_15120	Hypothetical protein (Tfp pilus assembly protein PilX)[F]	3.18	1.60E-06
AD56_15110	Hypothetical protein (Tfp pilus assembly protein PilV)[F]	3.01	0.026
AD56_15115	Hypothetical protein (Tfp pilus assembly protein PilW)[F]	3.00	3.02E-06
AD56_09685	Hypothetical protein (Tfp pilus assembly protein, major pilin PilA)[F]	2.58	0.003
AD56_01075	Type-1 fimbrial protein, A chain[D]	2.40	1.08E-15
AD56_16440	AI-2 transport protein TqsA[I]	2.36	1.11E-05

Table S6.7 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
AD56_15130	Fimbrial protein[F]	2.36	8.16E-04
AD56_01070	Putative fimbrial chaperone YadV[J]	2.31	0.039
AD21_05660	Type II secretion system protein E[E]	-0.99	6.79E-04
AD56_15125	Type IV pilus biogenesis factor PilY1[E]	2.30	5.12E-20
AD56_03025	Fimbrial protein[F]	2.14	0.022
AD56_10480	Hypothetical protein (Tfp pilus assembly protein, major pilin PilA)[F]	1.89	0.001
AD56_04130	Antigen 43[L]	1.87	1.93E-11
AD56_17355	Poly-beta-1,6-N-acetyl-D-glucosamine synthase	1.83	0.021
AD56_01065	Outer membrane usher protein HtrE[J]	1.81	0.042
AD56_00365	AI-2 transport protein TqsA[I]	1.55	0.047
AD56_03170	Hypothetical protein (Putative hemolysin)[K]	1.55	0.001
AD56_21565	Type IV pilus biogenesis and competence protein PilQ[E]	1.42	0.011
AD56_14665	GTP-binding protein TypA/BipA[M]	1.36	4.19E-07
AD56_15365	Putative diguanylate cyclase YcdT[J]	1.17	0.037
AD56_03030	Fimbrial protein	1.15	0.001
AD56_07875	Cellulose synthase catalytic subunit [UDP-forming]	1.05	0.002
AD56_07905	Cyclic di-GMP-binding protein[H]	0.97	0.034
AD56_05795	Adhesin YadA	0.93	2.41E-07
AD56_09930	Type IV pilus biogenesis factor PilY1[E]	0.90	0.020
AD80_17030	S-fimbrial protein subunit SfaA	1.58	0.014
AD80_19590	Hypothetical protein (Flp pilus assembly protein CpaB)[C]	-1.78	1.03E-09
AD80_25255	Hypothetical protein (Pilus assembly protein TadC)[A]	-1.31	0.001
AD80_08405	Putative diguanylate cyclase YcdT[J]	-1.30	0.004
AD80_16255	Type 4 prepilin-like proteins leader peptide-processing enzyme[F]	-1.17	5.38E-04
AD80_25295	Type II secretion system protein D[E]	-1.11	3.80E-04
AD80_25285	Hypothetical protein (Flp pilus assembly protein TadG)[A]	-1.10	5.08E-04
AD80_26280	Fimbrial protein[F]	-1.10	1.69E-05
AD87_27855	Biofilm operon icaADBC HTH-type negative transcriptional regulator IcaR	-6.66	0.008
AD87_13885	Putative diguanylate cyclase YcdT[J]	-3.35	4.53E-06
AD87_20505	Putative pyruvyl transferase EpsO	-2.36	1.21E-06
AD87_13620	AI-2 transport protein TqsA[I]	-2.21	2.55E-04
AD87_30350	AI-2 transport protein TqsA[I]	-1.89	0.003
Ter91_09750	S-fimbrial protein subunit SfaA	3.91	5.17E-07
Ter91_09755	Putative fimbrial chaperone LpfB[J]	3.36	0.006
Ter91_13975	Putative fimbrial chaperone YadV[J]	2.32	1.45E-10
Ter91_14155	Type II secretion system protein E[E]	2.30	1.13E-07
Ter91_14165	Type II secretion system protein G[E]	2.01	3.28E-11
Ter91_19995	GTP-binding protein TypA/BipA[M]	1.91	1.44E-30

Table S6.7 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
Ter91_13065	Hypothetical protein (Flp pilus assembly protein TadB)[A]	1.88	7.22E-05
Ter91_13970	Outer membrane usher protein FimD[D]	1.83	2.67E-19
Ter91_14420	Putative biofilm formation methyltransferase WspC	1.80	6.66E-05
Ter91_13080	Type IV pilus biogenesis and competence protein PilQ[E]	1.78	2.29E-10
Ter91_13085	Hypothetical protein (Flp pilus assembly protein CpaB)[C]	1.70	8.92E-06
Ter91_23580	Putative type II secretion system protein HxcR[E]	1.58	0.013
Ter91_13060	Hypothetical protein (Pilus assembly protein TadC)[A]	1.54	0.003
Ter91_11165	Hypothetical protein (Tfp pilus assembly protein PilZ)[F]	1.33	0.001
Ter91_13965	Fimbria adhesin protein	1.23	1.14E-06
Ter91_19975	Antigen 43[L]	0.90	0.006
Ter91_15510	Type II secretion system protein E[E]	0.86	0.008
Ter91_04775	Hypothetical protein (Pilus assembly protein TadC)[A]	0.74	5.12E-04
Ter91_02550	Hypothetical protein (Tfp pilus assembly protein PilP)[F]	0.62	0.029

[1] AD21, *Pseudomonas sp.*; bAD24, *Burkholderia sp.*; AD56, *Dyella sp.*, AD80, *Janthinobacterium sp.*; AD87, *Paenibacillus sp.*; Ter91, *Collimonas pratensis*.
[2] COG description is indicated and used for further identification of the function of the gene classified as hypothetical proteins..
[3] Log2 Fold change.
[4] False discovery rate.
[A] Part of Tad machine that assembles Type IVb pili required for adhesion to abiotic surfaces (Bernard et al. 2009).
[B] Part of Type IVb pili required for adhesion to abiotic surfaces (Bernard et al. 2009).
[C] Encode for Type IV pilus biogenesis apparatus (Tomich et al. 2007) that is involved in biofilm formation and twitching motility (Burdman et al. 2011).
[D] Involved in biogenesis of Type 1 pilus that can facilitate adhesion to biological surfaces (Schilling et al. 2001).
[E] Part of Type II secretion system that delivers matrix proteins for biofilm formation (Johnson et al. 2014).
[F] Part of Type IV pilus (Wall & Kaiser 1999) that involved in biofilm formation and twitching motility (Burdman et al. 2011).
[G] Involved in biosynthesis of alginate as extracellular matrix to aid biofilm formation (Hay et al. 2013).
[H] Synthesizes the signaling molecule cyclic dimeric GMP which binds the PilZ domain of the cellulose synthase BcsA, activating production of cellulose as biofilm matrix (Cowles et al. 2016).
[I] Controls the transport of the quorum-sensing signal AI-2 and the biofilm formation (Herzberg et al. 2006).
[J] Part of the yadCKLM-htrE-yadVN fimbrial operon. Could contribute to adhesion to various surfaces in specific environmental niches (Korea et al. 2010).
[K] Can be required for biofilm formation and adhesion (Caiazza & O'Toole 2003).
[L] May function as an adhesin (Hasman et al. 1999).
[M] Can be involved in biofilm formation (Neidig et al. 2013).

Table S6.8. Differentially expressed genes of bacteria related to nitrogen and carbon uptake in presence of *F. culmorum* in rhizospheric soil after four days of incubation. Up-regulated and down-regulated genes in fungal presence are indicated by log2FC > 0 and log2FC < 0, respectively.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Nitrogen assimilation		
AD21_09800	Urocanate hydratase[A]	3.09	3.24E-55
AD21_09805	Putative allantoin permease[A]	1.57	2.91E-05
AD21_01715	Histidine transport ATP-binding protein HisP[A]	0.91	0.004
AD21_09830	Histidine ammonia-lyase[A]	0.61	0.048
AD21_04695	Glutamine transport ATP-binding protein GlnQ	-3.53	1.78E-08
AD21_04700	Glutamine transport system permease protein GlnP	-2.89	1.89E-04
AD21_18315	Glutamine transport ATP-binding protein GlnQ	-2.47	2.47E-09
AD21_08700	Glutamine transport ATP-binding protein GlnQ	-2.47	2.91E-18
AD21_08690	Glutamine transport system permease protein GlnP	-2.31	1.10E-13
AD21_18305	Histidine-binding periplasmic protein	-2.17	1.33E-07
AD21_00065	Nitrogen assimilation regulatory protein (ntrC)[B]	-1.92	0.002
AD21_04690	ABC transporter glutamine-binding protein GlnH	-1.74	1.43E-07
AD21_26120	Glutamine synthetase	-1.62	0.005
AD21_09825	Histidine ammonia-lyase[A]	-1.32	1.09E-05
AD21_02285	Glutamine transport ATP-binding protein GlnQ	-1.24	7.58E-08
AD21_10290	Glutamate synthase [NADPH] small chain	-1.17	1.45E-06
AD21_26490	Imidazolonepropionase[A]	-1.20	0.001
AD21_08030	Nitrogen assimilation regulatory protein (ntrC)[B]	-1.13	2.03E-10
AD21_15125	Glutamine transport ATP-binding protein GlnQ	-1.05	9.83E-10
AD21_08805	Glutamine-dependent NAD(+) synthetase	-1.04	0.003
AD21_12975	Nitrogen assimilation regulatory protein (ntrC)[B]	-0.96	7.46E-10
AD21_11095	Histidine transport ATP-binding protein HisP	-0.91	0.001
AD21_05230	Nitrogen regulatory protein P-II[C]	-3.53	1.15E-10
bAD24_chr1_14065	Glutamate dehydrogenase	3.84	4.25E-72
bAD24_chr1_18595	Proton glutamate symport protein	2.99	6.51E-09
bAD24_chr1_04290	Histidine transport system permease protein HisQ	2.27	0.008
bAD24_chr1_14075	putative glutamine ABC transporter permease protein GlnM	1.75	1.46E-11
bAD24_chr1_04215	Histidine-binding periplasmic protein	1.57	1.23E-09
bAD24_chr1_14070	Glutamate/aspartate periplasmic-binding protein	1.51	9.35E-18
bAD24_chr1_14080	Glutamate/aspartate transport system permease protein GltK	1.18	2.91E-04
bAD24_chr1_14085	Glutamine transport ATP-binding protein GlnQ	1.16	1.10E-04
bAD24_chr1_04280	Histidine transport ATP-binding protein HisP	0.97	0.008
bAD24_chr1_04495	Histidine ammonia-lyase[A]	0.96	0.008
bAD24_chr1_06740	Nitrogen regulatory protein P-II[C]	-1.35	2.09E-08
bAD24_chr1_18785	Nitrogen regulation protein NR(I) (ntrC)[B]	-1.08	8.71E-05
AD56_04105	Imidazolonepropionase[A]	2.66	9.16E-06
AD56_19375	Histidine ammonia-lyase[A]	2.26	0.001
AD56_19705	Urocanate hydratase[A]	2.16	1.61E-08
AD56_23230	NAD-specific glutamate dehydrogenase	1.74	2.08E-15

Table S6.8 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Nitrogen assimilation		
AD56_11375	Nitrogen regulation protein NR(I) (ntrC)[B]	1.07	0.008
AD56_13190	Glutamine synthetase	2.10	4.77E-04
AD56_01170	Glutamate synthase [NADPH] large chain	-1.96	2.30E-12
AD56_01175	Glutamate synthase [NADPH] small chain	-1.84	9.36E-06
AD80_00480	Glutamate dehydrogenase	1.98	2.89E-07
AD80_18765	Nitrogen regulatory protein P-II[C]	1.60	8.41E-06
AD80_17850	Ferredoxin-dependent glutamate synthase 1	1.41	2.26E-17
AD80_22100	Nitrogen regulation protein NR(ntrC)[B]	1.16	9.80E-04
AD80_11185	Imidazolonepropionase[A]	-1.68	6.46E-07
AD80_05865	Nitrogen regulatory protein P-II[C]	-1.16	1.97E-04
AD80_10550	Nitrogen regulation protein NR(II) (ntrB)[B]	-0.90	5.57E-04
AD87_11365	Glutamine transport system permease protein GlnP	-3.52	8.98E-04
AD87_27805	Glutamate synthase [NADPH] small chain	-1.74	1.32E-09
AD87_07330	Glutamine synthetase	-1.68	3.84E-11
AD87_17840	Glutamine synthetase	-1.19	2.67E-05
Ter91_21630	ABC transporter glutamine-binding protein GlnH	6.97	1.48E-26
Ter91_23205	Glutamate dehydrogenase	3.19	2.98E-15
Ter91_16765	Glutamine transport system permease protein GlnP	3.17	0.016
Ter91_24205	Nitrogen regulatory protein P-II[C]	3.05	1.85E-24
Ter91_03800	Proton glutamate symport protein	2.79	9.78E-09
Ter91_16775	Glutamine transport ATP-binding protein GlnQ	2.64	7.93E-04
Ter91_20500	Nitrogen regulation protein NR(II) (ntrB)[B]	2.62	9.60E-07
Ter91_14555	Urocanate hydratase[A]	2.07	2.43E-07
Ter91_20495	Nitrogen regulation protein NR(I) (ntrC)[B]	2.02	8.05E-06
Ter91_01050	Glutamate/aspartate periplasmic-binding protein	2.00	2.23E-05
Ter91_23180	Glutamate/aspartate transport system permease protein GltK	1.46	4.69E-05
Ter91_23200	Glutamate/aspartate periplasmic-binding protein	1.40	1.99E-08
Ter91_02775	Ferredoxin-dependent glutamate synthase 1	1.36	1.10E-12
Ter91_23190	Glutamate/aspartate periplasmic-binding protein	1.20	2.54E-11
Ter91_02780	Glutamate synthase [NADPH] small chain	1.18	0.003
Ter91_04805	Nitrogen assimilation regulatory protein (ntrC)[B]	1.02	0.020
	Carbon source		
AD21_13420	Trehalose transport system permease protein SugB	4.17	2.58E-30
AD21_13425	Sn-glycerol-3-phosphate transport system permease protein UgpA	4.11	5.85E-27
AD21_18365	Sn-glycerol-3-phosphate transport system permease protein UgpA	3.54	6.96E-33
AD21_18375	Sn-glycerol-3-phosphate import ATP-binding protein UgpC	2.67	4.22E-19
AD21_05520	Glycerol-3-phosphate transporter	1.13	3.40E-05
AD21_12740	Trehalose transport system permease protein SugB	-2.14	0.002
AD21_12735	Trehalose transport system permease protein SugA	-1.91	0.006
AD21_21550	Sn-glycerol-3-phosphate import ATP-binding protein UgpC	-1.00	0.002

Table S6.8 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Carbon source		
bAD24_chr1_14865	Trehalose transport system permease protein SugA	3.75	1.11E-19
bAD24_chr1_14870	Trehalose transport system permease protein SugB	3.04	1.07E-08
bAD24_chr2_13555	HTH-type transcriptional regulator CdhR[D]	2.16	3.67E-05
bAD24_chr2_07810	Sn-glycerol-3-phosphate import ATP-binding protein UgpC	1.88	0.044
bAD24_chr2_08680	Ribose transport system permease protein RbsC	1.67	7.31E-04
bAD24_chr2_08685	D-ribose-binding periplasmic protein	1.60	8.62E-08
bAD24_chr1_07065	Sn-glycerol-3-phosphate transport system permease protein UgpA	0.87	1.73E-04
bAD24_chr1_07055	Trehalose import ATP-binding protein SugC	0.71	0.010
bAD24_chr1_01735	Trehalose transport system permease protein SugB	-3.25	0.002
bAD24_chr1_01725	Sn-glycerol-3-phosphate import ATP-binding protein UgpC	-2.59	6.06E-07
AD56_17685	HTH-type transcriptional regulator CdhR[D]	4.36	0.049
AD56_13880	HTH-type transcriptional regulator CdhR[D]	1.93	0.019
AD80_09740	Trehalose transport system permease protein SugA	2.08	0.006
AD80_26115	Trehalose transport system permease protein SugB	-3.29	8.15E-06
AD80_26105	Sn-glycerol-3-phosphate import ATP-binding protein UgpC	-3.10	1.63E-06
AD87_13380	Trehalose operon transcriptional repressor	-6.58	0.002
AD87_23840	Trehalose import ATP-binding protein SugC	-1.42	1.92E-09
Ter91_18780	Trehalose transport system permease protein SugA	7.41	1.05E-09
Ter91_18785	Trehalose transport system permease protein SugB	7.12	1.33E-23
Ter91_07590	Ribose import ATP-binding protein RbsA	4.35	9.93E-23
Ter91_07595	Ribose transport system permease protein RbsC	3.60	1.62E-07
Ter91_08170	Ribose transport system permease protein RbsC	3.28	0.006
Ter91_06695	HTH-type transcriptional regulator CdhR[D]	2.95	1.49E-17
Ter91_07600	D-ribose-binding periplasmic protein	2.65	9.67E-06
Ter91_14580	Ribose import ATP-binding protein RbsA	2.58	0.050
Ter91_16570	Trehalose import ATP-binding protein SugC	2.22	7.86E-14
Ter91_16560	Sn-glycerol-3-phosphate transport system permease protein UgpA	2.20	3.36E-07
Ter91_11755	D-ribose-binding periplasmic protein	1.44	2.55E-04
Ter91_18220	Sn-glycerol-3-phosphate-binding periplasmic protein UgpB	1.44	0.017

[1] AD21, *Pseudomonas sp.*; bAD24, *Burkholderia sp.*; AD56, *Dyella sp.*, AD80, *Janthinobacterium sp.*; AD87, *Paenibacillus sp.*; Ter91, *Collimonas pratensis*.

[2] COG description is indicated and used for further identification of the function of the gene classified as hypothetical proteins..

[3] Log2 Fold change.

[4] False discovery rate.

[A] Involved in the degradation of histidine as alternative nitrogen source (Yu et al. 2006; Stopnisek *et al.* 2016).

[B] Member of the two-component regulatory system NtrB/NtrC involved in the activation of nitrogen assimilatory genes such as glutamine synthetase gene glnA (Alvarez-Morales et al. 1984).

[C] Indirectly controls the transcription of glnA and activate glutamine synthetase under nitrogen limitation (Huergo et al. 2003).

[D] Induces the transcription of the PA5384-PA5388 operon in response to carnitine. This operon is involved in the degradation of L-carnitine. (Wargo & Hogan 2009).

Table S6.9. Differentially expressed genes of bacteria related to secondary metabolite production, defense mechanisms, and aromatic or volatile compound degradation in presence of *F. culmorum* in rhizospheric soil after four days of incubation. Upregulated and downregulated genes in fungal presence are indicated by log2FC > 0 and log2FC < 0, respectively.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Secondary metabolite production		
AD21_14020	ncRNA RsmY[A]	0.94	0.003
AD21_21285	Hydroxyneurosporene desaturase[B]	0.80	0.004
AD21_20115	Aclacinomycin methylesterase RdmC[C]	-2.55	1.36E-05
AD21_12515	Oxygen-dependent dichlorochromopyrrolate synthase[D]	-1.65	1.45E-05
AD21_23645	Linear gramicidin synthase subunit D[‡]	-1.58	1.31E-03
AD21_00200	Linear gramicidin synthase subunit B[‡]	-1.53	9.43E-07
AD21_00435	4,5-DOPA dioxygenase extradiol[E]	-1.52	2.81E-04
AD21_17780	15-cis-phytoene desaturase*	-1.44	4.83E-06
AD21_09090	Levodione reductase[F]	-1.35	6.66E-05
AD21_26020	Linear gramicidin synthase subunit D[‡]	-1.29	0.004
AD21_24850	Linear gramicidin synthase subunit B[‡]	-1.27	2.03E-04
AD21_24860	Tyrocidine synthase 1[‡]	-1.19	0.001
AD21_23005	NADPH-dependent curcumin reductase	-1.13	5.23E-04
AD21_23650	Linear gramicidin synthase subunit B[‡]	-1.08	0.003
AD21_05805	Phospholipase YtpA[V]	-0.92	0.011
AD21_25920	Aminopyrrolnitrin oxygenase PrnD[G]	-0.88	3.27E-04
bAD24_chr1_02665	All-trans-phytoene synthase*	0.99	6.78E-05
bAD24_chr1_16170	Phospholipase YtpA[V]	0.80	0.033
AD56_01750	Hypothetical protein (Proline 4-hydroxylase)[H]	2.58	0.014
AD56_15685	Hypothetical protein (Proline 4-hydroxylase)[H]	2.06	0.006
AD56_17035	Metalloprotease PmbA[I]	2.00	5.70E-07
AD56_18000	Linear gramicidin synthase subunit D[‡]	1.96	0.005
AD56_17240	Acyl carrier protein[J]	1.92	8.06E-04
AD56_17370	2-Aminobenzenesulfonate 2,3-dioxygenase subunit alpha	1.90	0.008
AD56_20435	Squalene--hopene cyclase*	1.77	0.002
AD56_05430	Acyl carrier protein[J]	1.75	0.045
AD56_20430	All-trans-phytoene synthase*	1.56	4.61E-04
AD56_00745	1-Deoxy-D-xylulose-5-phosphate synthase[K]*	1.55	0.002
AD56_21355	Putative bacilysin exporter BacE	1.55	0.003
AD56_10190	Polyketide synthase[J]	1.18	2.81E-04
AD56_18275	Acyl carrier protein[J]	0.99	0.003
AD56_10180	Tyrocidine synthase 3[‡]	0.88	4.33E-04
AD56_10185	Glutamate-1-semialdehyde 2,1-aminomutase 1[L]	0.80	0.025
AD87_26345	Levodione reductase[F]	-6.50	0.008
AD87_27870	Zeta-carotene-forming phytoene desaturase*	-2.64	0.002
AD87_16695	Tetraprenyl-beta-curcumene synthase*	-2.45	2.56E-04
AD87_05735	1-Deoxy-D-xylulose-5-phosphate synthase[K]*	-1.64	6.19E-06

Table S6.9 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Secondary metabolite production		
Ter91_21420	Hypothetical protein (Ca2+-binding RTX toxin-related protein)	3.05	7.96E-07
Ter91_14900	All-trans-phytoene synthase*	2.50	0.008
Ter91_14910	15-cis-phytoene desaturase*	2.49	0.002
Ter91_14905	All-trans-phytoene synthase*	2.22	6.53E-04
Ter91_11495	Acyl carrier protein[J]	2.07	2.36E-04
Ter91_07935	Acyl carrier protein[J]	2.02	1.58E-29
Ter91_09580	Tyrocidine synthase 3[‡]	1.79	5.17E-06
Ter91_09585	Linear gramicidin synthase subunit B	1.74	0.003
Ter91_11490	Acyl carrier protein[J]	1.56	0.011
Ter91_18570	Linear gramicidin synthase subunit D[‡]	1.54	1.46E-06
Ter91_11815	Dimodular nonribosomal peptide synthase	1.48	4.86E-07
Ter91_11450	Colicin V production protein	1.24	0.001
	Defense mechanisms		
AD21_24230	Multidrug resistance protein MdtN	-3.40	1.35E-07
AD21_11255	Multidrug export protein EmrB	-3.10	0.003
AD21_19225	Streptothricin hydrolase[M]	-2.57	1.78E-08
AD21_16240	Multidrug resistance protein MdtB	-2.40	9.56E-11
AD21_19260	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	-2.36	7.77E-14
AD21_19255	Hypothetical protein (Type VI protein secretion system component VasF)[X]	-2.26	1.18E-11
AD21_14420	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	-2.25	5.92E-04
AD21_19245	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	-2.20	6.49E-13
AD21_19250	Hypothetical protein Type VI protein secretion system component VasK)[X]	-2.15	1.23E-13
AD21_19295	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	-2.06	4.45E-14
AD21_19270	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	-2.05	8.10E-18
AD21_19290	Hypothetical protein (Type VI protein secretion system component VasA)[X]	-2.04	9.88E-19
AD21_25455	Chitinase D	-2.04	1.32E-16
AD21_19275	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	-2.02	1.04E-17
AD21_22350	Serralysin[N]	-1.99	5.23E-06
AD21_19280	Hypothetical protein (Type VI protein secretion system component Hcp, secreted cytotoxin)[X]	-1.91	9.65E-15
AD21_14415	Hypothetical protein (Type VI protein secretion system component VasF)[X]	-1.89	0.004
AD21_05010	Putative multidrug resistance protein EmrK	-1.86	2.87E-18

Table S6.9 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Defense mechanisms		
AD21_28000	Macrolide export protein MacA	-1.74	0.001
AD21_16245	Multidrug resistance protein MdtA	-1.64	1.63E-09
AD21_19315	Hypothetical protein (Implicated in type VI secretion and phage assembly)[X]	-1.66	7.23E-13
AD21_24825	Multidrug export protein EmrB	-1.57	4.04E-05
AD21_04675	Macrolide export protein MacA	-1.56	2.19E-08
AD21_12585	Multidrug resistance protein MdtC	-1.55	1.54E-04
AD21_05565	Multidrug resistance protein MdtN	-1.55	2.72E-05
AD21_13600	Serralysin[N]	-1.51	1.23E-09
AD21_14410	Hypothetical protein (Type VI protein secretion system component VasK)[X]	-1.45	1.27E-06
AD21_03925	Streptothricin hydrolase[M]	-1.41	0.003
AD21_25450	Chitin-binding protein CbpD	-1.40	3.48E-04
AD21_25475	Streptothricin hydrolase[M]	-1.39	5.88E-09
AD21_13625	Extracellular serine protease	-1.34	0.002
AD21_06020	Putative 4-amino-4-deoxy-L-arabinose-phosphoundecaprenol flippase subunit ArnE[O]	-1.34	0.003
AD21_04680	Multidrug resistance protein MdtE	-1.31	1.58E-07
AD21_06285	Multidrug resistance protein MdtA	-1.31	1.77E-09
AD21_24630	Multidrug export protein MepA	-1.29	1.74E-05
AD21_14395	Hypothetical protein (Implicated in type VI secretion and phage assembly)[X]	-1.24	0.002
AD21_13605	Alkaline proteinase inhibitor	-1.20	2.56E-04
AD21_11405	Extracellular serine protease	-1.16	8.22E-17
AD21_20380	Putative multidrug resistance protein EmrK	-1.09	3.53E-06
AD21_07535	Multidrug resistance protein MexB	-1.06	5.60E-05
AD21_06280	Multidrug efflux pump subunit AcrB	-1.02	3.19E-12
AD21_26885	Multidrug resistance protein MdtL	-0.93	2.18E-06
AD21_18330	Putative multidrug export ATP-binding/permease protein	-0.90	2.38E-04
AD21_20375	Putative multidrug resistance protein EmrY	-3.40	0.002
AD21_01370	Putative multidrug export ATP-binding/permease protein	-3.10	1.21E-05
bAD24_chr1_02995	Multidrug resistance protein NorM	1.59	0.009
bAD24_chr1_07385	Multidrug resistance protein MdtB	1.46	0.005
bAD24_chr1_08785	HTH-type transcriptional regulator TtgR[P]	1.44	7.68E-11
bAD24_chr1_03860	GTP-binding protein TypA/BipA[Q]	1.08	7.96E-11
bAD24_chr1_12935	Daunorubicin/doxorubicin resistance ATP-binding protein DrrA	0.96	1.26E-04
bAD24_chr1_04160	Multidrug resistance protein MdtA	0.84	8.82E-04
bAD24_chr1_01935	Multidrug resistance protein MdtA	-6.29	0.005
bAD24_chr2_04760	Putative multidrug-efflux transporter	-3.16	7.25E-12
bAD24_chr1_07775	Multidrug export protein EmrB	-2.90	1.69E-04
bAD24_chr1_16405	Multidrug resistance protein NorM	-1.94	0.002
bAD24_chr1_15765	Toxin HigB-1	-1.72	1.11E-06

Table S6.9 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Defense mechanisms		
bAD24_chr2_07960	Multidrug resistance operon repressor	-1.69	9.14E-05
bAD24_chr2_10250	Hypothetical protein (Type VI protein secretion system component Hcp, secreted cytotoxin)[X]	-1.66	0.002
bAD24_chr2_10245	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	-1.66	3.34E-08
bAD24_chr1_01180	Hypothetical protein (Phospholipase/lecithinase)	-1.64	0.002
bAD24_chr2_11290	Multidrug export protein EmrB	-1.59	7.02E-05
bAD24_chr2_00740	Multidrug resistance protein MdtA	-1.29	1.88E-05
bAD24_chr2_10260	Hypothetical protein (Type VI protein secretion system component VasA)[X]	-1.21	5.44E-04
bAD24_p_00350	Multidrug resistance protein MdtA	-1.13	0.005
bAD24_chr1_04300	Putative multidrug-efflux transporter	-1.03	7.25E-12
bAD24_chr2_08030	Multidrug export protein EmrB	-1.00	1.69E-04
bAD24_chr2_08015	Multidrug resistance protein NorM	-0.99	0.002
bAD24_chr2_13450	Putative bifunctional chitinase/lysozyme	-6.29	0.001
AD56_17795	Hypothetical protein (Type VI protein secretion system component Hcp, secrete cytotoxin)[X]	3.78	5.19E-08
AD56_22995	Putative bifunctional chitinase/lysozyme	3.42	2.23E-05
AD56_17810	Hypothetical protein (Type VI protein secretion system component VasA)[X]	3.25	1.49E-04
AD56_17785	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	2.84	3.75E-08
AD56_17845	Hypothetical protein (implicated in type VI secretion)[X]	2.81	1.70E-08
AD56_17920	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	2.80	1.56E-04
AD56_17790	Hypothetical protein (Predicted component of the type VI protein secretion system)[X]	2.65	1.52E-14
AD56_06565	Hypothetical protein (Multidrug resistance efflux pump)	2.70	0.008
AD56_07605	Alkaline phosphatase synthesis transcriptional regulatory protein PhoP[R]	2.61	0.037
AD56_14150	Multidrug resistance protein MdtB	2.42	0.016
AD56_12595	Beta-lactamase	2.23	0.014
AD56_23450	Outer membrane protein OprM[T]	2.17	1.08E-06
AD56_21635	Multidrug resistance protein MdtA	2.02	0.043
AD56_19625	Putative multidrug export ATP-binding/permease protein	1.95	0.001
AD56_05610	P-hydroxybenzoic acid efflux pump subunit AaeA	1.94	0.003
AD56_05720	Non-hemolytic phospholipase C[W]	1.92	0.040
AD56_03375	Multidrug resistance protein MdtC	1.79	0.010
AD56_06820	Virulence transcriptional regulatory protein PhoP[R]	1.69	1.35E-05
AD56_12850	Multidrug resistance protein MdtA	1.61	8.83E-04
AD56_23235	Multidrug resistance protein MexA	1.33	0.002
AD56_10260	Hypothetical protein (Lantibiotic modifying enzyme)	1.32	0.038

Table S6.9 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Defense mechanisms		
AD56_07690	Hypothetical protein (Uncharacterized conserved protein YvlB)[S]	1.23	0.039
AD56_01715	Outer membrane protein TolC[U]	1.22	1.81E-05
AD80_11205	Hypothetical protein (Antitoxin component YwqK of the YwqJK toxin-antitoxin module)	1.00	0.025
AD80_11200	Putative antitoxin YwqK	0.96	0.023
AD80_02085	Multidrug export protein EmrB	0.82	0.008
AD80_10060	Multidrug efflux pump subunit AcrB	-1.03	2.69E-04
AD80_10055	Multidrug resistance protein MexA	-1.00	1.31E-04
AD87_08415	Multidrug export protein MepA	2.38	5.93E-04
AD87_08705	Multidrug resistance operon repressor	-3.03	0.004
AD87_12435	Putative multidrug export ATP-binding/permease protein	-2.60	2.02E-04
AD87_01760	Multidrug resistance ABC transporter ATP-binding/permease protein BmrA	-2.30	9.69E-05
AD87_15125	Putative multidrug resistance ABC transporter ATP-binding/permease protein YheH	-1.81	3.43E-05
AD87_15120	Putative multidrug resistance ABC transporter ATP-binding/permease protein YheI	-1.69	4.85E-05
Ter91_06100	Extracellular serine protease	2.38	2.52E-32
Ter91_21905	Extracellular serine protease	4.01	6.88E-10
Ter91_10535	Putative bifunctional chitinase/lysozyme	3.09	3.57E-04
Ter91_09610	Multidrug resistance protein MdtB	2.70	1.10E-14
Ter91_06775	Macrolide export protein MacA	1.96	0.004
Ter91_06100	Extracellular serine protease	2.38	2.52E-32
Ter91_21905	Extracellular serine protease	4.01	6.88E-10
Ter91_10535	Putative bifunctional chitinase/lysozyme	3.09	3.57E-04
Ter91_04370	Alkaline phosphatase synthesis transcriptional regulatory protein PhoP[R]	1.96	6.72E-19
Ter91_17830	Multidrug resistance protein Stp	1.63	0.014
Ter91_20890	Outer membrane protein OprM[T]	1.60	0.020
Ter91_04640	Outer membrane protein TolC[U]	1.57	4.19E-06
Ter91_21335	Multidrug resistance protein MdtC	1.42	1.83E-08
Ter91_04620	Outer membrane protein OprM[T]	1.33	8.53E-05
Ter91_04625	Multidrug efflux pump subunit AcrB	1.29	3.91E-09
Ter91_15085	Outer membrane protein OprM[T]	1.15	2.67E-04
Ter91_15090	Multidrug export protein EmrA	1.02	0.026
Ter91_02815	Daunorubicin/doxorubicin resistance ATP-binding protein DrrA	0.99	9.50E-04
Ter91_16615	Macrolide export protein MacA	0.94	0.003
Ter91_13470	Phospholipase C2[W]	0.90	0.030
Ter91_14895	Multidrug resistance protein MdtA	0.89	0.007

Table S6.9 continued.

Sequence ID[1]	Gene description[2]	Log2FC[3]	FDR[4]
	Aromatic or volatile compound degradation		
AD21_28025	Anthranilate 1,2-dioxygenase large subunit	3.32	3.15E-38
AD21_06355	P-hydroxybenzoate hydroxylase	-2.87	3.75E-05
AD21_00055	Methanesulfonate monooxygenase	-2.45	3.73E-05
AD21_09210	Limonene 1,2-monooxygenase	-2.30	3.86E-11
AD21_11435	Pentachlorophenol 4-monooxygenase	-1.98	1.22E-16
AD21_00085	Haloacetate dehalogenase H-1	-1.88	4.54E-09
AD21_00025	2-Hydroxychromene-2-carboxylate isomerase	-1.46	0.001
AD21_21105	Alkanal monooxygenase alpha chain	-1.44	5.52E-06
AD21_04645	Dimethyl-sulfide monooxygenase	-1.43	0.005
bAD24_chr2_13385	Naphthalene 1,2-dioxygenase subunit alpha	2.27	2.52E-07
bAD24_chr2_05255	Naphthalene 1,2-dioxygenase/salicylate 5-hydroxylase systems, ferredoxin component	-1.27	4.67E-04
bAD24_chr2_13625	Dimethyl-sulfide monooxygenase	-1.23	3.23E-04
bAD24_chr1_00605	Muconate cycloisomerase 1	-1.03	0.004
bAD24_chr2_05310	Manganese-dependent 2,3-dihydroxybiphenyl 1,2-dioxygenase	-1.02	0.004
bAD24_chr1_18100	Benzoyl-CoA oxygenase component B	-0.85	2.25E-05
bAD24_chr2_07925	(S)-1-Phenylethanol dehydrogenase	-0.46	7.79E-04
bAD24_chr1_15985	Pentachlorophenol 4-monooxygenase	-0.35	0.001
bAD24_chr1_09235	Fumarylpyruvate hydrolase	-0.14	1.95E-05
AD56_00780	4-Formyl-benzenesulfonate dehydrogenase TsaC1/TsaC2	2.36	0.021
AD56_05985	Hypothetical protein (Dienelactone hydrolase)	2.12	0.018
AD56_00525	Hypothetical protein (Catechol 2,3-dioxygese or other lactoylglutathione lyase)	1.94	0.019
AD56_13865	Carboxy-methylene-butenolidase	0.63	0.046
AD80_24925	Muconate cycloisomerase 1	-2.75	1.99E-05
AD87_20640	Limonene 1,2-monooxygenase	-2.17	0.004
Ter91_01515	Carboxy-methylene-butenolidase	1.65	0.006
Ter91_04065	Salicylate 5-hydroxylase, large oxygenase component	1.40	7.47E-12
Ter91_07300	2-Hydroxymuconate semi-aldehyde hydrolase	0.77	0.020

[1] AD21, *Pseudomonas sp.*; bAD24, *Burkholderia sp.*; AD56, *Dyella sp.*, AD80, *Janthinobacterium sp.*; AD87, *Paenibacillus sp.*; Ter91, *Collimonas pratensis*.
[2] COG description is indicated and used for further identification of the function of the gene classified as hypothetical proteins.
[3] Log2 fold change.
[4] False discovery rate.
* Involved in terpene metabolism.
‡ Nonribosomal peptide synthase.
[A] RsmY and RsmZ, are key factors that relieve RsmA-mediated regulation of secondary metabolism (Valverde et al. 2003).
[B] Involved in the biosynthetic pathway of the carotenoid spheroidene (Albrecht et al. 1997).
[C] Involved in the biosynthesis of the anthracycline aromatic polyketide antibiotic aclacinomycin (Wang et al. 2000).
[D] Involved in the biosynthesis of the indolocarbazole rebeccamycin with antibacterial properties against gram-positive bacteria (Walisko et al. 2017).
[E] Involved in production of betalamic acid as structural unit of the betalains, natural nitrogen-containing water-soluble pigments with high colorant and bioactive properties (Gandia-Herrero & Garcia-Carmona 2014).
[F] Catalyzed the reversible oxidoreduction between levodione and actinol as chiral building blocks of optically active compounds such as xanthoxin (Sogabe et al. 2003).
[G] Involved in the biosynthesis of the antifungal antibiotic pyrrolnitrin (Kirner et al. 1998).

[H] Involved in non-ribosomal peptide formation. Hydroxyproline can be part of peptide antibiotics, such as actinomycin or etamycin (Shibasaki et al. 1999).
[I] Involved in maturation and secretion of Microcin B17 that is a ribosomaly synthesized peptide antibiotics (Allali et al. 2002).
[J] Acyl carrier proteins are components in polyketide biosynthesis (Zhou et al. 1999).
[K] Enzyme forms part of an alternative non-mevalonate pathway for terpenoid biosynthesis (Xiang et al. 2007).
[L] Enzyme participates in porphyrin biosynthesis (Luer et al. 2005).
[M] Catalyzes the hydrolysis of the amide bond of streptolidine lactam and thereby conferring resistance to streptothricin (Maruyama & Hamano 2009.
[N] Zn-endopeptidase that acts as a virulence factor to cause tissue damage (Park & Ming 2002).
[O] Both ArnE and ArnF are required for polymyxin resistance (Yan et al. 2007).
[P] Involved in the resistance to hydrophobic antibiotics such as chloramphenicol and tetracycline (Terán et al. 2003).
[Q] Involved in antimicrobial resistance (Neidig et al. 2013).
[R] PhoP–PhoQ is involved in regulating polymyxin resistance (Macfarlane et al. 1999).
[S] YvlB was identified as resistence gene against lipopeptide antibitoic daptomycin (Miller et al. 2013).
[T] The outer membrane component of the MexAB-OprM efflux system that contributes to antibiotic resistance (Zhao et al. 1998).
[U] Involved in export of antibiotics and other toxic compounds (Koronakis et al. 2000).
[V] Involved in the biosynthesis of the phospholipid antibiotic bacilysocin with especially antifungal activity (Tamehiro et al. 2002).
[W] Known as virulence factors (Titball 1993; König *et al.* 1997) which might be involved in disruption of fungal membrane and defense against competitors (Song *et al.* 2015b).
[X] Type VI secretion system (T6SS) is involved in intra- and interbacterial antagonism by delivering of toxins into both prokaryotic and eukaryotic cells (Russell et al. 2014; Journet & Cascales 2016).

Table S6.10. Up-regulated genes of bacteria in presence of the fungus *F. culmorum* PV after 4 days of incubation in rhizospheric soil. Differentially expressed genes obtained by edgeR V3.2 and DESeq2 Version 1.8.2 analysis are displayed.
See https://dataverse.nl/privateurl.xhtml?token=4e1a9c6a-3d86-4cd7-b9a8-1af9a7abcb78

Table S6.11. Down-regulated genes of bacteria in presence of the fungus *F. culmorum* PV after 4 days of incubation in rhizospheric soil. Differentially expressed genes obtained by edgeR V3.2 and DESeq2 Version 1.8.2 analysis are displayed.
See https://dataverse.nl/privateurl.xhtml?token=4e1a9c6a-3d86-4cd7-b9a8-1af9a7abcb78

Table S6.12. Up-regulated genes of bacteria in presence of the fungus *F. culmorum* PV after 6 days of incubation in rhizospheric soil. Differentially expressed genes obtained by edgeR V3.2 and DESeq2 Version 1.8.2 analysis are displayed.
See https://dataverse.nl/privateurl.xhtml?token=4e1a9c6a-3d86-4cd7-b9a8-1af9a7abcb78

Table S6.13. Down-regulated genes of bacteria in presence of the fungus *F. culmorum* PV after 6 days of incubation in rhizospheric soil. Differentially expressed genes obtained by edgeR V3.2 and DESeq2 Version 1.8.2 analysis are displayed.
See https://dataverse.nl/privateurl.xhtml?token=4e1a9c6a-3d86-4cd7-b9a8-1af9a7abcb78

Table S6.14. Expressed genes of *F. culmorum* PV in presence of bacteria after 4 days of incubation in soil supplied with artificial root exudates.
See https://dataverse.nl/privateurl.xhtml?token=4e1a9c6a-3d86-4cd7-b9a8-1af9a7abcb78

Table S6.15. Tentative volatile compounds produced by *F. culmorum* PV (F) or a bacterial community in fungal presence (BF) or absence (B) in rhizospheric soil. The bacterial community consisted of the bacteria *Burkholderia sp.* AD24, *Dyella sp.* AD56, *Collimonas pratensis* Ter91, *Janthinobacterium sp.* AD80, *Paenibacillus sp.* AD87, and *Pseudomonas sp.* AD21.

Treatment	Compound*	Compound class	RI
B	Butyl allyl ether (4)	Ether	784
	3-Heptanol (4, 6)	Alcohol	894
	4-Methyl-2-heptanone (4)	Ketone	941
	Unknown (4)	n.s.	946
	(E)-4-Undecene (6)	Alkene	1091
	Unknown (4, 6)	n.s.	1332
	2-(1,1-dimethylethyl)-1,4-Benzenediol (4)	Aromate	1508
	2,2,4-Trimethyl-1,3-pentanediol diisobutyrate (6)	Ester	1583
	Dibenzothiophene (4)	Aromate	1762
	Diisooctyl phthalate (6)	Aromate	2547
BF	1,3-Pentadiene (4, 6)	Alkene	545
	Hexane (6)	Alkane	602
	2,4,4-Trimethyl-1-pentene (6)	Alkene	717
	Methyl-isobutyl-ketone (6)	Ketone	745
	Cyclopentanone (6)	Ketone	793
	2-t-Butylfuran (6)	Aromate	850
	3-Heptanone (4, 6)	Ketone	882
	Unknown (4, 6)	n.s.	888
	3-Methyl-2-heptanone (4, 6)	Ketone	927
	6-Methyl-3-heptanone (4)	Ketone	940
	4-Octanone (4, 6)	Ketone	975
	Unknown (4)	n.s.	1025
	Camphenilone (4)	Terpene	1083
	Fenchone (4, 6)	Terpene	1088
	3-Ethyl-2-nonanone	Ketone	1159
	Unknown (4)	n.s.	1196
	Unknown (4)	n.s.	1241
	Unknown (4)	n.s.	1322
BF	Unknown (6)	n.s.	1423
	Unknown (6)	n.s.	1434
	Nonadecane (6)	Alkane	1904
F	3-Methyl-furan (4)	Aromate	614
	Unknown (6)	n.s.	1241
	Unknown (6)	n.s.	1407
	(+)-Acora-3,7(14)-diene (6)	Terpene	1419
	α-Cedrene (4, 6)	Terpene	1420
	Unknown (4)	n.s.	1422
	Cadina-1,4-diene (4)	Terpene	1479
	Unknown (6)	n.s.	1483
	3,5-di-tert-Butyl-4-hydroxybenzaldehyde (6)	Aromate	1759

Table S6.15 continued.

Treatment	Compound*	Compound class	RI
B and BF	(Z)-2-Buten-1-ol (6)	Alcohol	600
	2-Butanol (4, 6)	Alcohol	706
	3-Pentanol (4, 6)	Alcohol	715
	2,4,4-Trimethyl-1-pentene (4)	Alkene	717
	2,4-Dimethylfuran (4, 6)	Aromate	718
	2,2,3,3-Tetramethyl-butane (4, 6)	Alkane	727
	3-Methyl-2-pentanone (6)	Ketone	757
	3-Hexanone (6)	Ketone	787
	3-Ethyl-2-pentanone (6)	Ketone	834
	Unknown (4)	n.s.	842
	4-Methyl-2-hexanone (4)	Ketone	843
	4-Heptanone (6)	Ketone	868
	Cyclohexanol (4, 6)	Alcohol	884
	3-Ethyl-2,4-pentanedione (4)	Ketone	925
	4-Methyl-2-heptanone (4, 6)	Ketone	942
	Unknown	n.s.	946
	2-Octanone (4, 6)	Ketone	993
	1-Propenyl-benzene (4, 6)	Aromate	1003
BF and F	(E)-4-Undecene (4)	Alkene	1091
	4-Dimethyl-benzenemethanol (4, 6)	Aromate	1156
	5-Undecanone (6)	Ketone	1270
	Unknown aromatic compound (6)	Aromate	1297
	3-Octanone (4)	Ketone	989
	Decanoic acid methyl ester (4)	Ester	1325
	(Z)-Muurola-4(14),5-diene (4)	Terpene	1434
	Pentadecane (4, 6)	Alkane	1483
	t-Butylhydroquinone (6)	Aromate	1508
	3,3-Diethyltridecane (6)	Alkane	1650
	Unknown (6)	n.s.	1658
B, BF	(Z)-2-Buten-1-ol (4, 6)	Alcohol	600
and F	2-Pentanon (4, 6)	Ketone	661
	3-Pentanon (4, 6)	Ketone	700
	2,2,3,3-Tetramethyl-butane (6)	Alkane	727
	Pyridine (4, 6)	Aromate	755
	3-Methyl-2-Pentanone (4)	Ketone	757
	3-Hexanone (4, 6)	Ketone	787
	2-Hexanone (4)	Ketone	791
	3-Ethyl-2-Pentanone (4, 6)	Ketone	834
	4-Heptanone (4, 6)	Ketone	868
	2,6,6-Trimethyl-decane (4)	Alkane	1130
	5-Undecanone (6)	Ketone	1270
	Pentadecane (6)	Alkane	1483

n.s. not specified; RI Linear retention Index of a 30 × 0.25 mm ID RXI-5MS column.
* Numbers 4 and 6 indicate length of incubation: 4 and 6 days, respectively.

CHAPTER 7

Calling from Distance: Attraction of Soil Bacteria by Plant Root Volatiles

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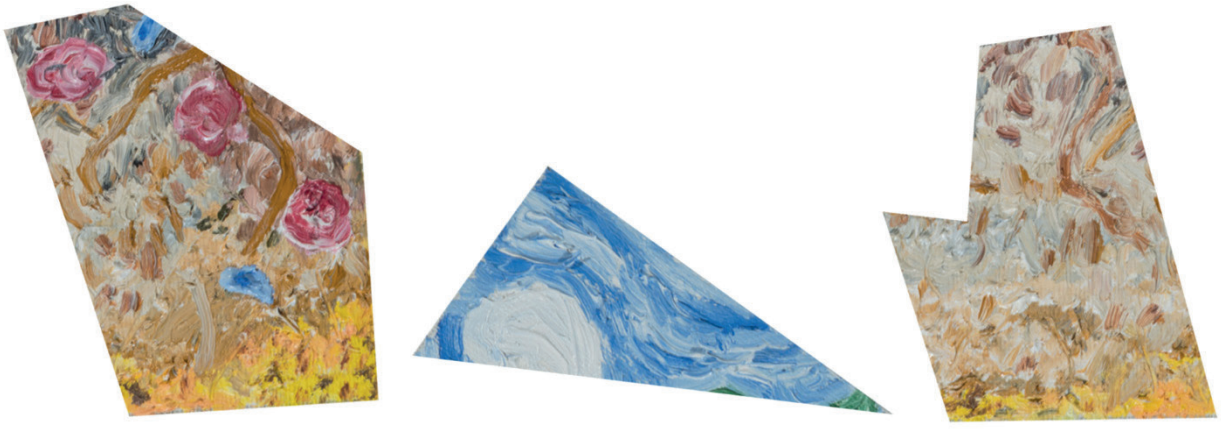


Abstract

Plants release a wide set of secondary metabolites including volatile organic compounds (VOCs). Many of those compounds are considered to function as defense against herbivory, pests and pathogens. However, little knowledge exists about the role of belowground plant VOCs for attracting beneficial soil microorganisms. We developed an olfactometer system to test the attraction of soil bacteria by VOCs emitted by *Carex arenaria* roots. Moreover, we tested whether infection of *C. arenaria* with the fungal pathogen Fusarium culmorum modifies the VOCs profile and bacterial attraction. The results revealed that migration of distant bacteria in soil towards roots can be stimulated by plant VOCs. Upon fungal infection, the blend of root VOCs changed and specific bacteria with antifungal properties were attracted. Tests with various pure VOCs indicated that those compounds can diffuse over long distance but with different diffusion abilities. Overall, this work highlights the importance of plant VOCs in belowground long-distance plant-microbe-interactions.

Keywords:

rhizosphere, plant-microbe interactions, synthetic microbial communities, volatiles, glass olfactometer

Introduction

Plants are exposed during their entire life to various environmental stresses such as water- and nutrient limitation, pathogenic infections and herbivory (Robert-Seilaniantz *et al.* 2010). Interactions with so-called beneficial or plant growth promoting microbes can relieve stresses (Mendes *et al.* 2013). Moreover, plants appear to be able to recruit those beneficial microbes to their roots (Paterson *et al.* 2007; Philippot *et al.* 2013; Rasmann & Turlings 2016). Plants produce a wide set of secondary metabolites (> 200,000 [Hartmann 2004]) and many of these metabolites play important role in belowground interactions, communication or recruitment of soil organisms. For instance, legumes produce flavonoids to attract nitrogen-fixing bacteria (Peters *et al.* 1986) and maize exudates benzoxazinoids to attract plant growth promoting bacteria such as *Pseudomonas putida* (Neal *et al.* 2012).

Besides soluble secondary compounds plants release various volatile organic compounds (VOCs) involved in interactions with surrounding soil organisms (Van Dam *et al.* 2016). It is estimated that VOCs constitute about 1 % of plant secondary metabolites (Venturi & Keel 2016). VOCs are small compounds of low molecular weight, lipophilic character, with high vapor pressure and low boiling points (Effmert *et al.* 2012; Bitas *et al.* 2013; Lemfack *et al.* 2014; Schmidt *et al.* 2015). Due to their physico-chemical properties, VOCs can easily diffuse through gas- and water-filled pores and can, therefore have a wide effective range in soil. Plant VOCs emitted by roots can function in multiple ways such as antimicrobials, food source, chemo-attractants or infochemicals (Van Dam *et al.* 2016). For example, root VOCs like (+)-enantiomers of carvone, limonene and borneol have been shown to promote bacterial quorum sensing (Ahmad *et al.* 2015). Furthermore, maize plants release the sesquiterpene (E)-β-caryophyllene in the rhizosphere when attacked by root feeding beetle larvae resulting in enhanced attraction of nematodes (Van Tol *et al.* 2001; Rasmann *et al.* 2005; Ali *et al.* 2010). Interestingly, a very fast emission of sesquiterpenes was also seen to be triggered in maize and other cereal plants upon infestation by *Fusarium spp.* (Piesik *et al.* 2011a; Piesik *et al.* 2011b; Becker *et al.* 2014) and terpenoid production of potato plants was affected by the inoculation with *Phytophthora infestans* (Henriquez *et al.* 2012).

However, so far it was not shown if plant VOCs such as terpenes can stimulate the motility of bacteria in the bulk soil. Moreover, it is unknown if plants can actively recruit beneficial soil microorganisms by emitting VOCs into the rhizosphere. Recent studies on microbial VOCs demonstrated that bacterial motility can be stimulated by VOCs (Garbeva *et al.* 2014a; Schmidt *et al.* 2016; Schulz-Bohm *et al.* 2016), although the applied test systems were rather artificial.

In this study, we tested the hypothesis that VOCs emitted by plants into the rhizosphere can attract soil bacteria from a long distance. To test this hypothesis, a glass

olfactometer system was designed to monitor bacterial migration in soil. With this system, we tested (1) if bacteria from a synthetic soil microbial community (originating from the rhizosphere of *Carex arenaria*) can be attracted to root VOCs emitted by *C. arenaria* and (2) whether there is a difference in the bacterial attraction when the roots of this plant were infected by the soil-borne fungal pathogen *Fusarium culmorum*. VOCs emitted from infected or non-infected roots were collected, analyzed and, in addition, the diffusion ability of some pure compounds in soil was examined.

The obtained results revealed for the first time that migration of distant soil bacteria outside the rhizosphere can be stimulated by plant root VOCs and that specific bacteria can be attracted by VOCs of plants infected by a fungal pathogen.

Material and Methods

Microorganisms and growth media

All bacterial strains (*Burkholderia* sp. AD024, *Collimonas pratensis Ter91*, *Dyella* sp. AD056, *Janthinobacterium* sp. AD080, *Paenibacillus* sp. AD087, and *Pseudomonas* sp. AD021) and the fungus *Fusarium culmorum* PV were isolated from the rhizosphere of *Carex arenaria* (Table S7.1) and cultured as described before (Schulz-Bohm *et al.* 2015; Schmidt *et al.* 2016; Schulz-Bohm *et al.* 2017a). Asexual spores (conidia) of *F. culmorum* were obtained by growing the fungus on Difco™ oatmeal agar plates (Becton, Dickinson and Company, France) with 0.2 mg ml^{-1} streptomycin for 3 weeks at 25°C. Collected spores were washed twice in sterile demineralized water (demi-water), counted and kept at -80°C until use.

Plants and cultivation

C. arenaria seeds were obtained from B and T World Seeds (Aigues-Vives, France). Prior use, the seed were surface sterilized with 70% ethanol and 4 g l^{-1} sodiumhypoclorite (bleach). In brief, seeds were incubated overnight in 150 ml demi-water containing 0.5 g glucose and 4 drops of Tween 80 to increase germination success. The next day, the seeds were washed three times for 1min in 70 % ethanol, two times with bleach and four times in sterile demi-water. Seeds were germinated on 0.5 Potato Dextrose Agar plates (Schmidt *et al.* 2016) for three weeks at 20°C. The *Carex* seedlings that appeared to be free of microbial contaminants (no bacterial or fungal growth was seen for the germinated seeds on PDA) were further cultivated for about three weeks of 16h at 22° C and 8h at 10°C day-night-cycle (100 µmol PAR m^{-2} s^{-1}) in pots filled with sterile glass-beads (1 mm diameter) and 0.5

Hoagland medium (590.4 µg ml^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 253.0 µg ml^{-1} KNO_3, 68.1 µg ml^{-1} KH_2PO_4, 246.5 µg ml^{-1} $MgSO_4 \cdot 7H_2O$, 2.9 µg ml^{-1} H_3BO_3, 1.8 µg ml^{-1} Mn Cl_2 $4H_2O$, 0.2 µg ml^{-1} $ZnSO_4 \cdot 7H_2O$, 0.1 µg ml^{-1} $CuSo_4 \cdot 5H_2O$, 0.1 µg ml^{-1} $Na_2MoO_4 \cdot 2H_2O$, and 41.5 µg ml^{-1} ferric EDTA). To stop the developing process the plants were kept at 4°C with a 10 h day-night-cycle. After about five weeks, seedlings were transferred from glass beads to gamma-sterilized soil (Schulz-Bohm *et al.* 2015) containing 14.3 µl per gram soil sterile demi-water, 13.5 µl per gram soil sterile P-buffer (18.4 mM KH_2PO_4, pH 6.5), 65.7 µl per gram soil sterile macronutrient stock solution (pH 6.5), and 0.9 µl per gram soil sterile micronutrient stock solution. The macronutrient stock consisted of 10.7 mg ml^{-1} MES, 1.6 mg ml^{-1} NH_4NO_3, 0.5 mg ml^{-1} K_2SO_4, 2.6 mg ml^{-1} $MgSO_4 \cdot 7H_2O$, and 0.3 mg ml^{-1} $CaCl_2 \cdot 2H_2O$. 50 ml of micronutrient stock solution consisted of 20 ml solution 1 (2.6 mg ml^{-1} H_3BO_3, 1.6 mg ml^{-1} $MnCl_2 \cdot 4H_2O$), 20 ml solution 2 (1.5 mg ml^{-1} $FeCl_2 \cdot 4H_2O$), 5 ml solution 3 (0.4 mg ml^{-1} $ZnCl_2$, 0.2 mg ml^{-1} $CuCl_2 \cdot 2H_2O$, 0.1 mg ml^{-1} $NiCl_2 \cdot 6H_2O$, 0.1 mg ml^{-1} $CoCl_2 \cdot 6H_2O$), and 5 ml solution 4 (13.0 mg ml^{-1} Na_2EDTA, 0.5 mg ml^{-1} $Na_2MO_4 \cdot 2H_2O$). *Carex* plants were incubated at 20°C and 16/8 h day-night-cycle (282 µmol PAR m^{-2} s^{-1}) and regularly watered with sterile demi-water.

Infection bioassay

Infection bioassays with *F. culmorum* were performed with 4-weeks old *C. arenaria* plants which were previously cultivated on glass beads. The plants were infected with *F. culmorum* by dipping the roots into 10^7 ml^{-1} spore suspension and planting it in *F. culmorum*-infected soil (9 % w/w moisture) which was pre-incubated for 4 days at 20 °C with 10^5 per gram soil spores, 0.45 mg per gram soil glucose, 12.0 µl per gram soil P-buffer (18.4 mM), 58.2 µl g^{-1} soil macronutrient stock solution, and 0.8 µl g^{-1} soil micronutrient stock solution. For the control treatment, demi-water instead of spore suspension was added. Plants were incubated at day-night-cycles of 16h at 22°C and 8h at 10°C (100 µmol PAR m^{-2} s^{-1}) and regularly watered with sterile demi-water. Infection symptoms were weekly monitored. After 5 weeks, plants were harvest to determine the shoot and root fresh weight.

Olfactometer system

To test the attraction of bacteria in soil to VOCs emitted by plant root or fungi, an olfactometer was developed (Coelen Glastechniek, Cuijk, The Netherlands). The olfactometer consisted of a central glass vessel (4.5 cm diameter, 6.5 cm high) and four rectangular glass-tubes (5.0 cm length [4.6 cm inner length] and 1.5 cm high [1.3 cm inner height]; Figure 7.2 A). The glass-tubes being open at the top to fill in soil and inoculate the

bacteria were connected to the central vessel (in 1.5 cm height from the bottom) via screw thread adapter couplings (4.0 cm length) with integral PTFE-faced silicone seals (DURAN Group GmbH, Wertheim/Main, Germany). The other side of the glass-tubes was closed with screw caps (with PTFE silicone seal, DURAN Group GmbH). The top of the glass-tube was closed with a glass lid fixed with parafilm. The connection part between the central vessel and the soil-filled part in the glass-tube had a length of about 4.5 cm (Figure 7.2 A). In between the central glass vessel and screw thread adapter couplings a nylon membrane of 1 µm mesh size (Plastok Associates Ltd, Birkenhead, UK) was placed to prevent potential passing of fungal hyphae or spores from soil in the central vessel into the glass-tubes.

Olfactometer bioassay

The set-up of the olfactometer bioassay consisted of four different treatments (Figure 7.2 A): Control (n = 3), *F. culmorum* (n = 3), *Carex* (n = 4) and *Carex* inoculated with *F. culmorum* (n = 4). The control treatments without plants and fungus consisted of 90 g soil mixed with 1.3 ml demi-water, 4.5 ml P-buffer (10 mM KH_2PO_4, pH 6.5), and 2.7 ml artificial root exudate solution (ARE, Schulz-Bohm *et al.* 2015). For the treatment with *F. culmorum*, 32.5 g *F. culmorum*-infected soil was pre-incubated for 10 days with 3 x 10^5 spores per gram soil, 0.95 mg per gram soil glucose, 0.04 ml per gram soil P-buffer and 0.01 ml per gram soil demi-water at 20°C. This soil was mixed with 60 g dry soil, 3.29 ml 10mM P-buffer and 2.7 ml ARE. The final carbon content for the treatments supplied with ARE was 222 µg C per gram soil. 5-weeks old *Carex* plants were treated or non-treated with *F. culmorum* and incubated for 5 days with the same day-night cycle conditions as described above. In brief, root tips of *Carex* plants were dipped in *F. culmorum* spore suspension (1.1 x 10^7 spores ml^{-1}) and planted into sterile hand-made nylon bags (0.5 mm mesh size, about 100 ml volume) containing 60 g soil mixed with macro- and micronutrients as described above and 32.5 g of *F. culmorum*-infected soil (see above) which was pre-incubated for 6 days at 20°C. For the non-treated plants, root tips of the *Carex* plants were dipped in sterile demi-water and planted in sterile nylon bags containing 90 g soil, demi-water, phosphate-buffer as well as macro- and micronutrients. The soil moisture in all central vessels was 9.4% w/w.

For each treatment, two of four glass-tubes connected to a central pot were filled with 10 g soil mixed with 0.5 ml P-buffer (10 mM KH_2PO_4, pH 6.5) and 0.3 ml ARE to obtain a final carbon concentration of 148 µg C per gram soil. The other two glass-tubes were filled only with soil (10 g) mixed with 0.8 ml P-buffer (Figure 7.2 A). At the end of each glass-tube (8.0 % w/w soil moisture), 100 µl of bacterial suspension consisting of six different bacteria was inoculated. Before, overnight cultures of the bacteria were set up (Schulz-Bohm *et al.* 2015)

and harvest by centrifuging for 10 min at 5,500 rpm and 18°C, followed by two-times washing steps with P-buffer and OD adjustment to obtain 10^8 CFU ml^{-1}. The bacterial suspensions were finally diluted to about 5 x 10^5 CFU ml^{-1} and mixed. After filling, closing and connecting all glass-tubes the nylon-bags with the soil and plants were added to the system. The olfactometer system was covered with aluminum foil (Figure S7.1) and incubated at 20°C with a 16/8h day-night-cycle.

After about 65 h, soil in distance of 0.8 cm from the connection point (Figure 7.2 A) was collected from each glass-tube and stored at -80°C till DNA extraction. Plants were harvest, carefully rinsed with water and scanned with an Epson Perfection V850 Pro Scanner. Furthermore, infected and non-infected parts of the root were microscopically visualized with Leica MD641 and Leica Application Suite Version 4.7.0 (Leica Microsystems B.V., Amsterdam, The Netherlands).

Volatile collection and analysis

VOCs released in the soil from *F. culmorum* or *Carex* roots were collected with steel traps filled with 150 mg Tenax TA and 150 mg Carbopack B (Markes International Ltd, Llantrisant, UK). The traps were connected to glass vessels (4.5 cm diameter and 6.5 cm height) with two outlets at the side (Figure S7.2). The vessels contained nylon bags filled with soil, soil and *F. culmorum*, or soil and *Carex* plants similar to the treatments described above. For each treatment, VOCs of four replicates were collected for 24 h simultaneously by two traps per vessel. Traps were removed, capped and stored at 4°C until GC-MS analysis (Supplementary Information, Appendix S1).

Diffusion assay of volatile pure compounds in soil

For testing the diffusion ability of pure volatile compounds a mix of VOCs representative for the VOCs blend produced by *Carex* roots and/ or *F. culmorum* was used (Table S7.2 and Table S7.4). A glass olfactometer system was set up with a central glass-vessel (4.5 cm diameter, 6.5 cm high) and two rectangular glass-tubes (12.5 cm length and 1.1 cm high) which were connected via screw thread adapter couplings (see above) to the central vessel (Figure 7.4 A). Both, central vessel and glass-tubes were covered with aluminum foil, which was fixed with parafilm to minimize evaporation of the volatile compounds. The central vessel contained 60 g soil mixed with sterile demi-water (9.4 % humidity [w/w]). 15 g soil was mixed with 10 mM P-buffer (8 % w/w humidity) and filled into the glass-tube. 0.55 ml of a mix of pure VOCs (about 5mM in 100 % methanol) was pipet in about 2 cm depth next to the connection points in the central vessel resulting in a final

VOCs concentration of 0.1 mM per gram soil. VOCs were collected with Rotilabo® -silicone tubes (PDMS-tubes; Carl Roth GmbH + Co. KG, Karlsruhe, Germany) which were placed into the soil of the central vessel as well as in the glass-tubes at five different distances (Figure 7.4 A). The PDMS tubes were pre-treated as described by Kallenbach *et al.* (2015). After 30 min or 60 min, PDMS tubes were removed and kept at -20°C until analysis. The experiment was replicated six times. To compare different diffusion abilities of the pure VOCs a relative peak area was calculated: Relative peak area = Peak area at distance Dn/ Peak area at distance D1.

DNA extraction and qPCR

DNA extraction was performed with DNeasy PowerSoil Kit (Qiagen Benelux B.V., Venlo, The Netherlands) as described in Supporting Information, Appendix S1. The DNA was quantified by NanoDrop and stored at -20°C until use.

All quantitative PCR (qPCR) were performed with a BioRad CFX96 C1000 Touch™ Thermal cycler according to the protocols described in Supplementary Information, Appendix S1. To transform qPCR data from 16S rRNA copy number into cell number, the 16S rRNA copy for each strain was determined, if not known from studies before (e.g. Song *et al.* 2015b), by qPCR of genomic DNA extracted from the same liquid culture where CFU were counted in parallel. The obtained cell numbers were corrected by the initial soil weight used for DNA extraction.

Statistics

Multivariate analysis of processed and normalized (log transformation and mean centered) GC-MS data was conducted with MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/MetaboAnalyst, Xia et al., 2015). VOCs were accounted as produced for each treatment when the average peak intensity for all replicates per treatment was at least 2-fold higher and significant different (Student's t-test, $P < 0.05$) from the controls.

Differences in bacterial abundance per treatment observed by qPCR and differences in relative peak areas of diffused VOCs were analyzed with R 3.1.1 (http://www.r-project.org/) using Anova followed by Tukey's HSD test (De Mendiburu 2014). Data was prior log- or square root-transformed to obtain normality of errors. Student's t-test was applied to analyze differences in the root and shoot biomass of *Carex* plants incubated with or without *F. culmorum*. Differences obtained by statistical tests were considered significant for $P < 0.05$.

Results

Infection of Carex arenaria by Fusarium culmorum PV

Inoculation of *C. arenaria* with the soil-borne fungus *F. culmorum* PV resulted in a significant reduction of plant biomass. The root and shoot weight of infested plants was about 7-times lower as compared to control plants (Table 7.1). Roots and leaves of almost all plants treated with the fungus were completely wilted after 5 weeks of incubation (Figure S7.3) demonstrating that *F. culmorum* can be pathogenic to *C. arenaria*. *Carex* plants used for testing the attraction of soil bacteria by root VOCs showed clear infection symptoms belowground (dark brown dots at roots) after eight days of incubation (Figure 7.1 and Figure S7.4). Furthermore, fungal growth was visible at the bottom of the shoot (Figure S7.4 L).

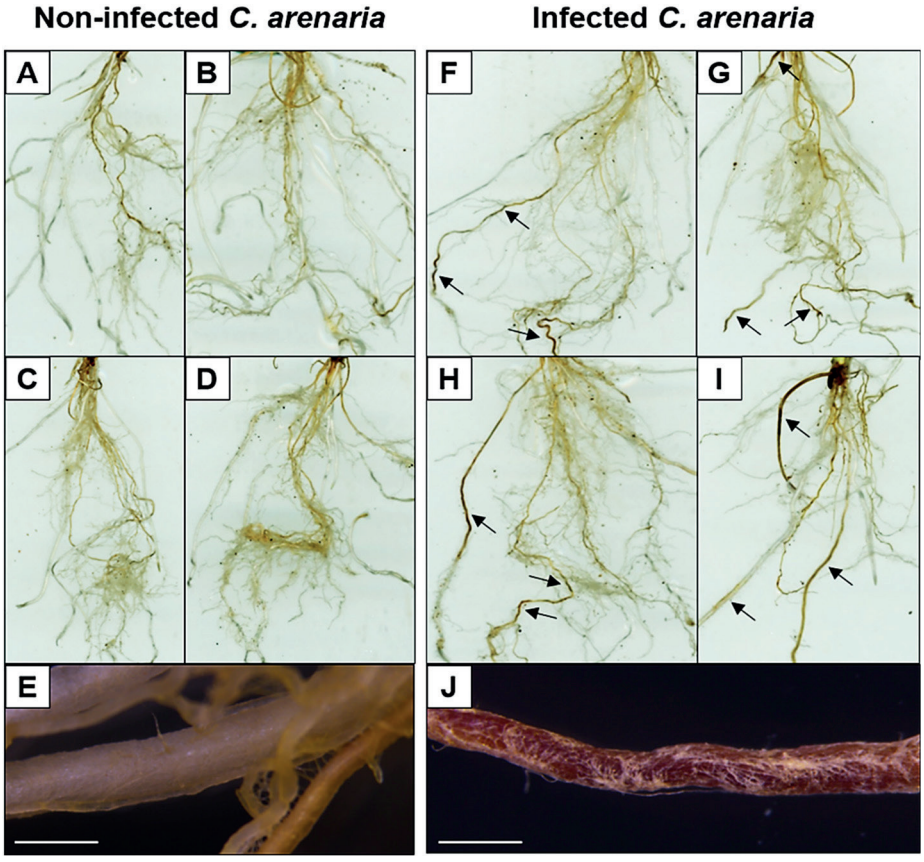
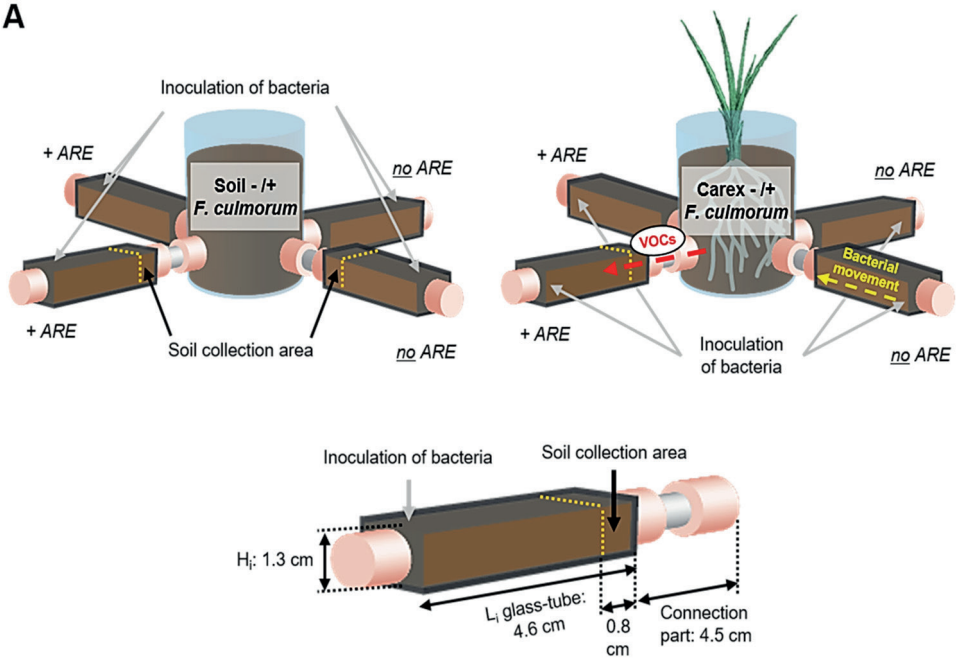


Figure 7.1. Roots of *Carex arenaria* growing for 8 days in absence (A-E) or presence of the soil-borne fungus *Fusarium culmorum* PV (F-J). Black arrows (F-I) indicate infection areas of the fungus at roots of *Carex* plants. Bars in panel E and J showing non-infected and infected root parts, respectively, represent 500 µm size.


A
Inoculation of bacteria
+ ARE
no ARE
Soil - /+
F. culmorum
+ ARE
Soil collection area
no ARE
+ ARE
no ARE
Carex - /+
F. culmorum
VOCs
Bacterial movement
+ ARE
Inoculation of bacteria
no ARE
Inoculation of bacteria
Soil collection area
Hi: 1.3 cm
Li glass-tube: 4.6 cm
0.8 cm
Connection part: 4.5 cm

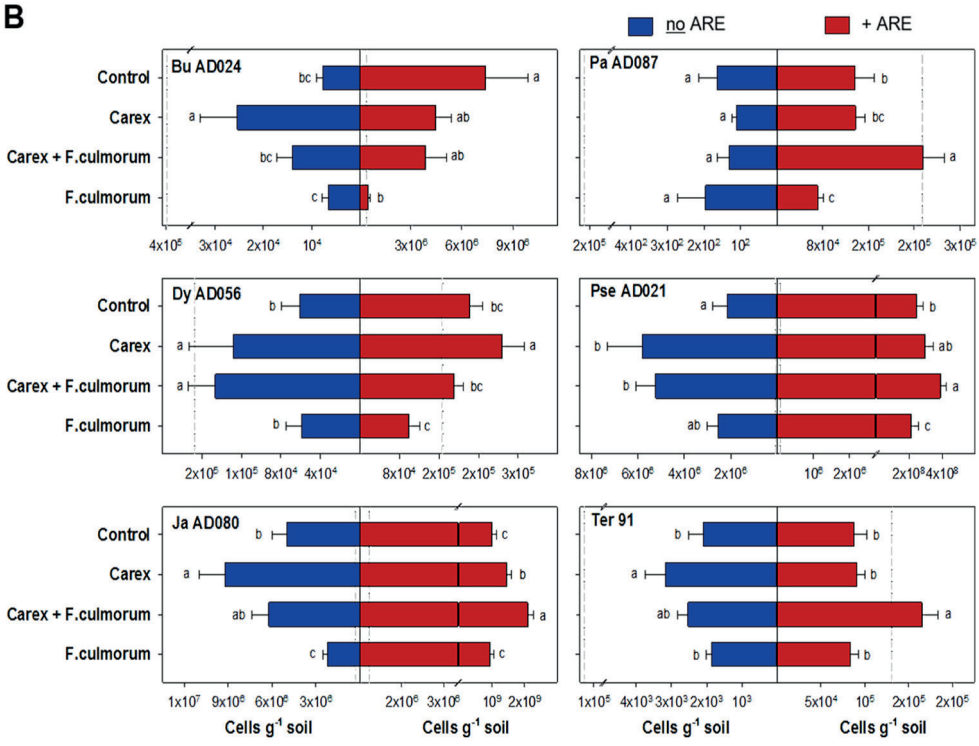

B
no ARE
+ ARE
Control
Carex
Carex + F.culmorum
F.culmorum
Bu AD024
Pa AD087
Dy AD056
Pse AD021
Ja AD080
Ter 91
Cells g-1 soil
Cells g-1 soil
Cells g-1 soil
Cells g-1 soil

Figure 7.2. Bacterial attraction towards VOCs in soil. (A) A glass olfactometer system with four different treatments in the central vessel: control (only soil); soil with *F. culmorum*; *Carex* and *Carex* with *F. culmorum*. Connected glass-tubes contained soil mixed with (+ ARE) or without artificial root exudates (no ARE). A synthetic bacterial community including *Burkholderia sp.* AD024, *Dyella sp.* AD056, *Janthinobacterium sp.* AD080, *Paenibacillus sp.* AD087, *Pseudomonas sp.* AD021, and *Collimonas pratensis* Ter91 was inoculated into soil at one end of the glass-tube. After 65 h soil was collected at 0.8 cm distance from the connection part to the central vessel (i.e. 3.8 cm distance from the inoculation point) (panel A below) to quantify the migrated bacteria. Drawing is not true to scale. Panel B, Bacterial number obtained by qPCR of the 16S rRNA gene at the collection area. 16S rRNA gene copy numbers were transformed into cell number based on the known copy number of the gene per genome. Different letters represent significant differences between the four treatments. Grey dotted line indicate initial bacterial concentration added at the inoculation point. Abbreviations: H_i, height of inner filling area; L_i, length of inner filling area; VOCs, volatile organic compounds.

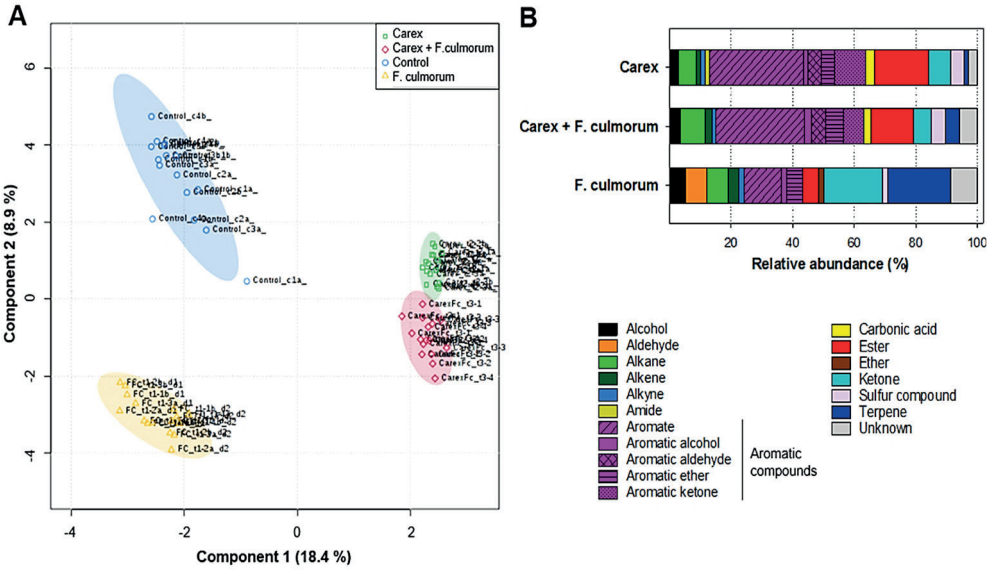


Figure 7.3. VOCs detected in the rhizosphere of *Carex arenaria* in presence or absence of *Fusarium culmorum* PV and by *F. culmorum* in soil. (A) sPLS-DA score plot of detected VOCs per treatment. Controls representing VOCs released from soil without fungus or plants are included in the analysis. (B) Relative abundance of identified VOCs per compound class (see legend) and treatment. Unknown: VOCs that could not be assigned with certainty to a known compound in the reference libraries.

Bacterial attraction by root VOCs of *C. arenaria*

To evaluate a potential stimulation of bacterial migration in soil, towards VOCs released by *F. culmorum* or by *C. arenaria*, an olfactometer system was set up (Figure 7.2 and Figure S7.1). In this system, bacteria inoculated at one end in a soil-containing glass-tube could migrate towards VOCs that were produced by *C. arenaria* or *F. culmorum* in the central vessel. Migrated bacteria in the glass-tubes were collected and quantified by qPCR. In the treatments with *C. arenaria,* the bacterial cell number per strain was up to three times higher as compared to the treatment consisting only of soil (Figure 7.2 B), indicating that volatiles emitted from plant roots can attract soil bacteria.

The stimulation of bacterial motility by plant VOCs released in presence or absence of *F. culmorum* was dependent on the access of nutrients. For example, stimulation of *Paenibacillus* was only observed by additional access to artificial root exudates while other bacteria (e.g. *Burkholderia*) were less attracted by the root VOCs in the presence of nutrients (Figure 7.2 B).

The cell numbers of bacteria inoculated in soil supplied with ARE was about 30 to 1000 times higher (with exception for *Dyella*) as compared to bacterial cell numbers in soil without ARE (Table S7.3). The number of migrated bacteria at the end of the glass-tubes filled with soil supplied with ARE were mostly above the amount of initial inoculum (Figure 7.2 B). The ratios of migrated cells towards *Carex* to migrated cells in the control were significant higher for *Burkholderia*, *Dyella*, and *Pseudomonas* (1.8-3.3) when they were inoculated in soil without ARE as compared to the conditions with access to ARE (0.5-1.6; Table S7.3).

Using the olfactometer approach we tested if the infection of *C. arenaria* with the fungal pathogen *F. culmorum* (Figure 7.1) affected the VOCs profiles (see below) and consequently bacterial attraction. For *Janthinobacterium*, *Collimonas* and *Paenibacillus* the numbers of migrated cells in soil supplied with ARE ($2.6x10^5$ - $2.1x10^9$ cells per gram soil) were significant higher for the *Carex* plants infected with *F. culmorum* as compared to non-infected *Carex* plants ($1.4x10^5$ - $1.5x10^9$ cells per gram soil) and to the control ($1.4x10^5$ - $3.3x10^8$ cells per gram soil; Figure 7.2 B and Table S7.3). This suggests that specific bacteria can be attracted to root volatiles of plants infected by a fungal pathogen. Interestingly, in the treatments inoculated only with *F. culmorum* without plants the number of migrated bacteria ($1.8x10^3$ - $2.5x10^6$ cells per gram soil without ARE and $7.1x10^4$ - $9.5x10^8$ cells per gram soil with ARE) was not different or even significant lower as compared to the control ($1.6x10^2$ - $2.2x10^6$ cells per gram soil without ARE and $8.8x10^4$ - $3.3x10^8$ cells per gram soil with ARE; Figure 7.2 B and Table S7.3). This indicates that VOCs emitted by the fungus in absence of *Carex* could not stimulate bacterial motility and some bacteria like *Burkholderia* and *Janthinobacterium* were repelled (Figure 7.2 B).

VOCs emission from the rhizosphere of infected and non-infected *C. arenaria* plants and *F. culmorum* in soil

A distinct blend of VOCs was emitted by different treatments (Table S7.4) and clear separations between the roots of *C. arenaria*, the fungus *F. culmorum*, and the control based on partial least squares discriminant analysis were observed (Figure 7.3 A). Although the VOCs profiles of *C. arenaria* in presence and absence of *F. culmorum* formed two distinct groups, these VOCs were more similar as compared to the VOCs profile of *F. culmorum* only (Figure 7.3). In total, most VOCs were detected for the treatments of *C. arenaria* inoculated with *F. culmorum* (86 versus 70 and 57 compounds for the treatments *Carex* and *F. culmorum*, respectively; Table S7.4). The identified VOCs released by infected or non-infected plant roots belonged to the classes of aromatic compounds (49-51 %), e.g. benzofuran or benzonitrile, and ester (14-17 %) such as γ-capro-, γ-deca or γ-nonalactone (Figure 7.3 and Table S7.4). Interestingly, in presence of the fungus *F. culmorum* more alkanes and the monoterpene (Z)-limonene-oxid were produced in the rhizosphere of *C. arenaria* (Figure 7.3 and Table S7.4). Most of the VOCs emitted by *F. culmorum* in soil were terpenes (21 %) such as α-pinene or camphene and ketones (19 %) like 2-nonanone or 3-octanone (Figure 7.3 and Table S7.4). In addition, several compounds were detected which could not be assigned with certainty to a classified volatile organic compound and remained unknown.

Diffusion of pure VOCs in soil

The diffusion of pure VOCs representative of *C. arenaria* or *F. culmorum* VOCs blend (Table S7.2) was measured in soil at various distances (Figure 7.4 A). Except for Amylene hydrate all VOCs were traceable until a distance of 12cm (Figure 7.4 B). For most compounds such as benzofuran or benzonitrile the detectable amounts decreased drastically with sampling distance. To compare the different diffusion abilities of the pure VOCs, a relative peak area in relation to the amount detected at the starting point was calculated. The VOCs propanal, γ-nonalactone and dimethyl disulfide had the highest relative peak areas (> 75 %; Table S7.5). Hence, some VOCs diffuse better over a long distance as compared to other compounds. The diffusion profile for α-pinene and camphene was very different, although these compounds belong to the same chemical class (monoterpenes) (Figure 7.4 B). At 12 cm distance, the traceable amount of α-pinene was about 30 times lower as compared to camphene (Table S7.5).

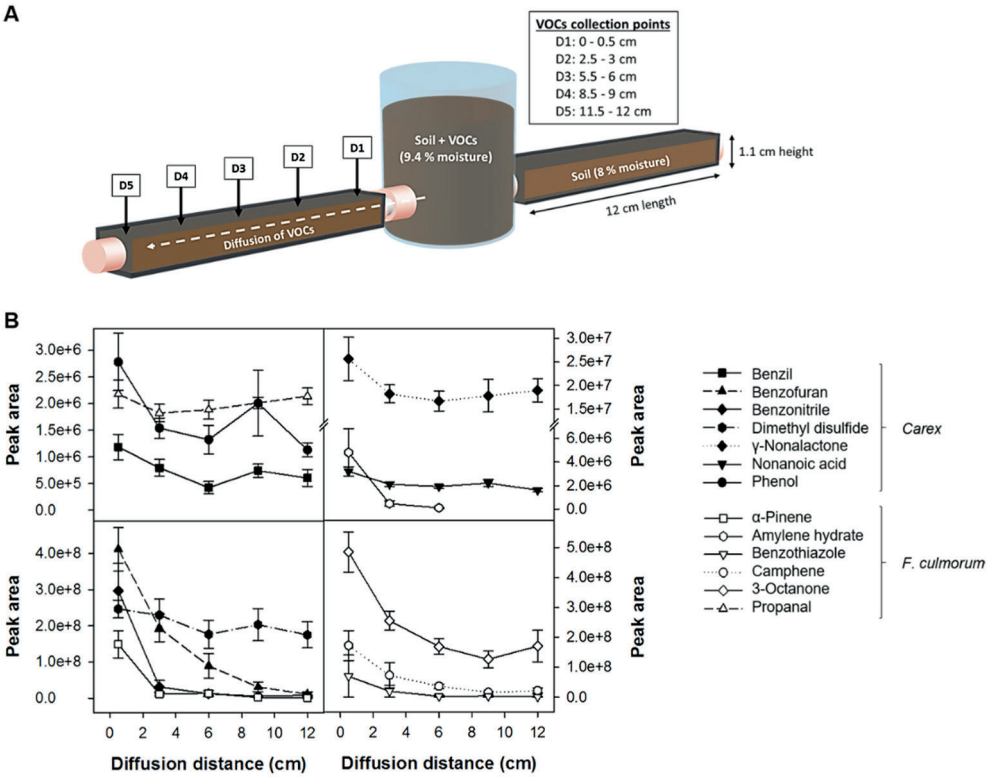


Figure 7.4. Diffusion of pure VOCs along a distance of 12 cm in soil. VOCs were collected with PDMS tubes for 30 min and 60 min at different distances in the connected glass-tubes 1 and 2, respectively (A). Data show mean values and standard errors (N = 6) for each synthetic compound after 60 min of collection (B). Filled and open symbols represent VOCs initially detected for *Carex* roots and *F. culmorum* in soil, respectively. A reliable detection directly at the release point in the central vessel was not possible because of surface saturation on the PDMS tubes.

Discussion

Plant release a wide range of secondary metabolites through their roots including VOCs. A main role of those compounds is considered to be a defense as they are often used to fight off herbivory, pests and pathogens (Selvaraj 2015; Venturi & Keel 2016). Although VOCs are generally considered as easily diffusible compounds in the soil matrix and important for belowground plant interactions (Van Dam *et al.* 2016) limited knowledge exist about the role of plant VOCs for attracting beneficial soil organisms.

Here, we developed a glass olfactometer system in order to test the attraction of soil bacteria from a synthetic community to VOCs emitted by the roots of *C. arenaria* or by the plant-pathogenic fungus *F. culmorum*. Olfactometer systems have been used successfully to study aboveground plant-herbivories interactions (Smith *et al.* 1993; Ballhorn & Kautz 2013) or belowground plant-nematode interactions (e.g. Rasmann *et al.* 2005). However, this is the first case to apply an olfactometer to study plant-microbe interactions.

Here, we revealed for the first time that VOCs emitted by plant roots play an important role in the long-distance attraction of bacteria from bulk soil. Interestingly, the addition of nutrients to the bulk soil in form of ARE influenced the VOC-mediated plant-bacteria interactions. For example, *Burkholderia*, *Dyella* and *Pseudomonas* were attracted less by root VOCs in the presence of nutrients while *Paenibacillus* was attracted only when additional nutrients were added to the bulk soil. These results suggests that under nutrient limitation certain bacteria might be intensely attracted by plant VOCs. Hence, plant VOCs can act as info-chemicals providing information about a nearby nutrient-rich environment. It is plausible that some plant VOCs such as terpenes can function as a direct source of nutrients as reported previously (Van Dam *et al.* 2016), explaining the increase in cell number of *Janthinobacterium* and *Pseudomonas* during the incubation in nutrient-poor bulk soil.

Using the olfactometer system, we tested how the infection with the pathogenic fungus *F. culmorum* affects the attraction of the soil bacteria. *F. culmorum* is an important fungal plant pathogen that causes diseases in a wide diversity of cereal and non-cereal crops and wild plants including *C. arenaria* (Scherm *et al.* 2013). Our results revealed that indeed some bacteria of the synthetic community such as *Janthinobacterium, Collimonas* and *Paenibacillus* were more attracted to VOCs of infected plants. This indicates that plants can recruit specific bacteria upon biotic stress situations. Direct interaction assays between *F. culmorum* and *Janthinobacterium, Collimonas* and *Paenibacillus* showed that those bacteria could successfully inhibit fungal growth (Figure S7.5). Moreover, previous studies demonstrated that mycophageous *Collimonas* strains have antifungal activity against various soilborne pathogenic fungi (Leveau *et al.* 2010), produce a range of antifungal compounds (Song *et al.* 2015b), and can successfully decrease fungal infections of tomato

plants (Kamilova *et al.* 2007). Similar results were reported for biocontrol strains of *Paenibacillus* (Grady *et al.* 2016). The GC-MS analysis revealed that different blends of VOCs were produced by roots of *C. arenaria* as compared to *F. culmorum*. VOCs emitted by *C. arenaria* where mainly composed of aromatic compounds and esters. It is plausible that some of those compounds can be involved in plant defense against pathogens. For instance, benzofuran and benzofuran as well as acetophone, benzaldehyde or nonanoic acid were previously shown to be root exudates components with antifungal activity (Spencer & Towers 1991; Curir *et al.* 2000; Utama *et al.* 2002; Jang *et al.* 2012; Khanam & Shamsuzzaman 2015). Besides fighting against potential pathogens, specific root VOCs of *C. arenaria* can be involved in the attraction of distant bacteria. For example, it was reported that the ester γ-caprolactone can enhance the colonization of potato roots by *Rhodococcus* or *Pseudomonas* when applied in a hydroponic system (Cirou *et al.* 2011). Also for dimethyl disulfide despite its antifungal activity (Kanchiswamy *et al.* 2015a) an attraction of soil organisms such as nematodes has been reported previously (Horiuchi *et al.* 2005) and it can stimulate bacterial growth (Garbeva *et al.* 2014a). This suggests that same VOCs can fulfil different functions depending on the interacting partner.

Fungal infection affected the VOCs profile of *C. arenaria* and led to the emission of VOCs such as the monoterpene (Z)-limonene-oxid. Interestingly, an induction of terpene and terpenoid production upon fungal infection was previously observed for various plants (Piesik *et al.* 2011a; Piesik *et al.* 2011b; Henriquez *et al.* 2012; Becker *et al.* 2014), indicating that those compounds might play an important role in plant-pathogene-interactions or attraction of beneficial bacteria important for plant protection.

The fungus *F. culmorum* emitted a specific blend of VOCs dominated by terpenes. Recently, it was demonstrated that those VOCs play an important role in the long-distance interaction with bacteria by affecting both motility and production of secondary metabolites (Schmidt *et al.* 2016; Schmidt *et al.* 2017). Here, VOCs of *F. culmorum* did not significantly stimulated bacterial motility and function as repellence as in the case of *Burkholderia* or *Janthinobacterium*. In accordance to previously reported antibacterial activity of fungal VOCs (Ngan *et al.* 2012; Keri *et al.* 2015; Schmidt *et al.* 2016), the compounds benzothiazole or terpenes including 1,8-cineole and camphene emitted by *F. culmorum* in soil might played a role in the inhibition of bacterial motility and growth.

Several VOCs produced by the roots of *C. arenaria* or *F. culmorum* were tested in a diffusion assay revealing that VOCs of different classes can disperse over long distance (> 12 cm) in soil. This supports the hypothesis that VOCs can indeed play a significant role in long-distance belowground interactions.

Overall, this study provides novel information on the ecological importance of VOCs in plant-microbe-interactions in soil. We demonstrated for the first time that distant soil bacteria can be attracted by plant root VOCs and revealed that plant VOCs emitted under a stress situation such as fungal infestation can recruit beneficial bacteria from outside the rhizosphere. In contrast to the vast majority of studies on VOC-mediated belowground interactions using very artificial conditions and studying only one-to-one interactions (reviewed in Bitas *et al.* 2013; Schmidt *et al.* 2015; Selvaraj 2015), the olfactometer setting used in this study resembles more closely the natural conditions belowground. Thus, the obtained results can strongly contribute to the understanding of the VOC-mediated interactions in an ecosystem context and pinpoint that VOCs are important signals or antimicrobials with potential for application in agriculture.

Acknowledgments

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Author Contributions

KSB and PG contributed to conception and experiments designed. KSB performed all experiments in collaboration with MH SG, and JM. KSB analysed and interpreted the data as well as created figures and tables. KSB and PG drafted the manuscript with contributions from all co-authors.

Supplementary Material 7.1 (Material and Methods)

Table S7.1. Bacterial and fungal strains used in this study. All strains were previously isolated from the rhizosphere of *Carex arenaria* or *Ammophila arenaria* growing on sandy dune soils at different places of the Netherlands.

Bacterial & fungal strains (Accesion number)	Origin	Phylum/ Division (Family)
Burkholderia sp. AD024 (PRJNA320371)	Dune grassland near Ouddorp, Zeeland *(De Ridder-Duine et al. 2005)*	Beta-Proteobacteria *(Burkholderiaceae)*
Collimonas pratensis Ter91 (CP013234)	Inner coastal dune soil in Terschelling *(De Boer et al. 2004)*	Beta-Proteobacteria *(Oxalobacteraceae)*
Dyella sp. AD056 (KJ685269)	Drift sand near Loon op Zand, Brabant *(De Ridder-Duine et al. 2005)*	Gamma-Proteobacteria (*Xanthomonadaceae*)
Janthinobacterium sp. AD080 (KJ685292)	Coastal outer dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	Beta-Proteobacteria *(Oxalobacteraceae)*
Paenibacillus sp. AD087 (LXQN00000000)	Pine plantation near Loon op Zand, Brabant *(De Ridder-Duine et al. 2005)*	Firmicutes (Paenibacillaceae)
Pseudomonas sp. AD021 (DQ778036)	Coastal inner dunes of Midsland, Terschelling *(De Ridder-Duine et al. 2005)*	Gamma-Proteobacteria *(Pseudomonadaceae)*
Fusarium culmorum PV	Dutch Coastal foredunes (*De Rooij-van der Goes et al. 1995*)	Ascomycota (*Nectriaceae*)

Table S7.2. Pure volatile compounds used in the volatile diffusion assay. Compounds were detected for roots of *Carex arenaria* (Carex) in absence or presence of the fungus *Fusarium culmorum* (Carex + FC) as well as *F. culmorum* (FC) in soil.

Compound	CAS number	RI‡	Exact mass (Da)§	Compound class	Treatment
α-Pinene*	80-56-8	937±3	136	Terpene	FC
Amylene hydrate (2-Methyl-2-butanol)*	75-85-4	628±4	88	Alcohol	FC
Benzil (Diphenyl-ethanedione)*	134-81-6	1766±2	210	Aromatic ketone	Carex/ Carex + FC
Benzofuran (Coumarone)*	271-89-6	1004±4	118	Aromatic ether	Carex + FC
Benzonitrile*	100-47-0	985±4	103	Aromatic compound	Carex/ Carex + FC
Benzothiazole*	95-16-9	1229±8	135	Sulfur compound	FC
Camphene*	79-92-5	952±2	136	Terpene	FC
Dimethyl disulfide*	624-92-0	746±6	94	Sulfur compound	Carex/ Carex + FC
γ-Nonalactone (Dihydro-5-pentyl- 2(3H)-Furanone)*	104-61-0	1363±5	156	Ester	Carex/ Carex + FC
Nonanoic acid*	112-05-0	1273±7	158	Carbonic acid	Carex/ Carex + FC
3-Octanone*	106-68-3	986±3	128	Ketone	FC
Phenol†	108-95-2	980±4	94	Aromatic compound	Carex/ Carex + FC
Propanal (Propionaldehyd)*	123-38-6	461±18	58	Aldehyde	FC

* Purchased from Sigma-Aldrich Chemie N.V. (Zwijndrecht, The Netherlands).
† Purchased from VWR International (Radnor, USA).
‡ Obtained from NIST 2014 V2.20 spectral library.
§ Experimental Retention Index for (semi-) standard non-polar columns notified in the NIST 2014 spectral library.

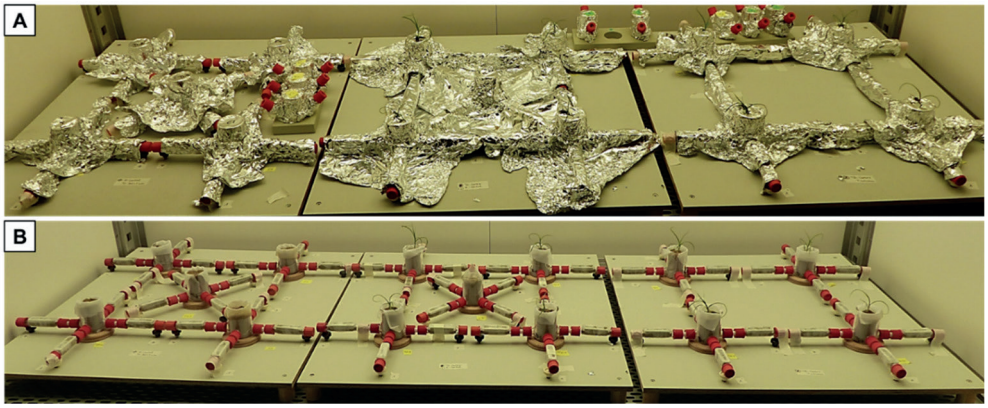

Figure S7.1. Set-up of the belowground olfactometer system to test the bacterial attraction by *Carex arenaria* root volatiles or volatiles emitted by the fungus *Fusarium culmorum* PV in soil. The system was covered with aluminium foil (A) to protect the soil from light above. For treatments with *C. arenaria* in the central vessel, four replicates were set up and three replicates were set up for the other treatments (B).

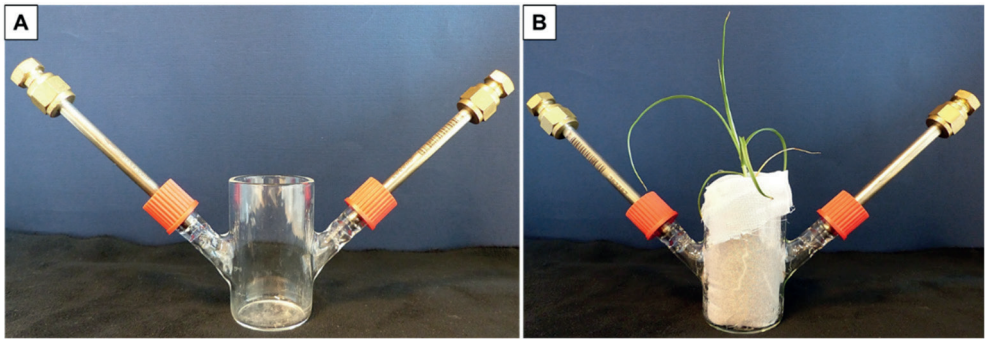

Figure S7.2. Experimental system to collect volatiles produced by plant roots in the rhizosphere.

GC-MS analysis of VOCs

VOCs were desorbed from the traps or PDMS tubes (see below) by using an automated thermodesorption unit (model UnityTD-100, Markes International Ltd., UK) at 250°C for 12 min (He flow 50 ml/min). The desorbed VOCs were subsequently collected on a cold trap at -10°C and introduced into the GC-QTOF (model Agilent 7890B GC and the Agilent 7200AB QTOF, USA) by heating the cold trap for 10 min to 280°C. A split ratio was set to 1:10 and 1:2 to analyze VOCs produced by *Carex* or *F. culmorum* in soil and diffused pure volatile compounds, respectively. The column used was a 30 × 0.25 mm ID DB-5MS with as film thickness of 0.25 µm (Agilent 122-5532, USA). The temperature program was as follows: 2 min at 39°C, 3.5°C/min to 95°C, 4°C/min to 165°C and finally 15°C/min to 280°C that was hold for 15 min. VOCs were detected by the MS operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (30–400 AMU, 4 spectras/sec). Collected GC/MS data was converted to mzData files using the Chemstation B.06.00 (Agilent Technologies, Santa Clara, USA) and further processed (peak picking, baseline correction and peak alignment) in an untargeted manner with MetAlign (Lommen & Kools 2012) and MSClust (Tikunov *et al.* 2012).

Detected compounds were identified by using NIST-MS Search by comparing the spectra, accurate mass, linear retention indices and spectra match factor with NIST 2014 V2.20 (National Institute of Standards and Technology, USA, http://www.nist.gov), Wiley 9th edition, and in-house spectral libraries. The linear retention indices of VOCs were calculated according to the method of Strehmel *et al.* (2008). Chromatograms obtained for the mix of introduced pure VOCs in soil (see below) were analyzed with Chemstation B.06.00 and AMDIS 2.72 (National Institute of Standards and Technology, Gaithersburg, USA).

DNA extraction and qPCR

DNA extraction was performed with DNeasy PowerSoil Kit (Qiagen Benelux B.V., Venlo, The Netherlands) with modification from the manufacturer's protocol. About 0.3-0.35 g soil was weighted in PowerBead Tubes and mixed by vortexing with 100 µl low molecular weight salmon sperm DNA (10 mg ml^{-1}, pH 8, Sigma-Aldrich Chemie N.V., Zwijndrecht, The Netherlands) as well as 60 µl solution C1 provided by the kit. The tubes were incubated for 30 min at 60°C and vortexed in a Vortex Adapter for 15 min at maximum speed. The solutions was spin down at 10,000 g for 1 min and the supernatant was mixed in a fresh collection tube with solution C2 and C3 followed by an incubation for 5 min on ice. The following steps were performed according to the protocol. The DNA was quantified by NanoDrop and stored at -20°C until use.

All quantitative PCR (qPCR) of the 16S rRNA genes of five of the six bacterial strains was performed according to Schulz-Bohm *et al.* (2015). The 20 µl reaction mixture to amplify

16S rRNA genes of *C. pratensis* Ter91 consisted of 1-fold SensiFAST™ SYBR® No-ROX Kit (GC biotech B.V., Alphen aan den Rijn, The Netherlands), BSA (0.5 µg µl-1), 375 nM forward and reverse primers (Eddy3for and Eddy3rev; Höppener-Ogawa *et al.* 2007), and 5 µl of DNA (2-6 ng μl^{-1}). No template controls consisting of DNase- and RNase-free water were included in every qPCR run. The thermal cycling program was as followed: 5 min initial denaturation at 95°C , ensued by 40 cycles of denaturation for 30 s at 95°C, annealing for 20 s at 62°C, elongation for 20 s at 72°C, and fluorescence signal detection for 15 s at 77°C. Immediately after the 40th PCR cycle, a melting curve analyses from 62°C to 95°C with increments of 1.0°C was followed.

Direct interaction assay

A fungal plug (1 cm diameter) with *Fusarium culmorum* PV (Table S7.1) pre-grown on 0.5 PDA (Schmidt *et al.* 2016) was transferred to the top of a petri-dish which was filled with 20 ml 0.1 TSA (Tyc *et al.* 2014) and incubated for 24 h at 25°C. Bacterial suspensions (10^8 CFU ml^{-1}) of *Burkholderia* sp. AD024, *Collimonas pratensis Ter91*, *Dyella* sp. AD056, *Janthinobacterium* sp. AD080, *Paenibacillus* sp. AD087, and *Pseudomonas* sp. AD021 (Table S7.1) were prepared as described before (main text). 50 µl of bacterial suspension per strain and, in case of the control, 50 µl of sterile P-buffer (10mM KH_2PO_4, pH 6.5) was spread in the middle of the agar plate. Petri-dishes were closed with parafilm and incubated for 5 days at 25°C. Pictures of the agar plate were taken with a Panasonic DMC-FZ200 digital camera.

Supplementary Material 7.2 (Supporting Results)

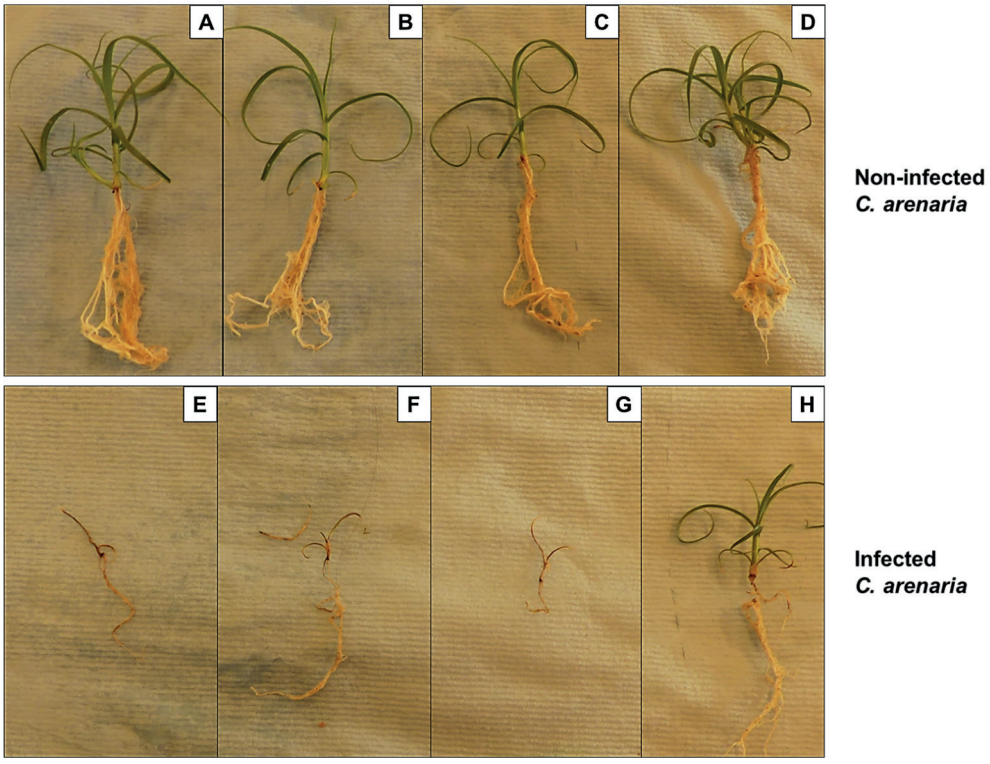

Figure S7.3. *Carex arenaria* after 5 weeks of incubation without (A-D) or with (E-H) *Fusarium culmorum* PV in soil. Most infected *Carex* plants showed clear wilting symptoms at shoots and roots (E-G).

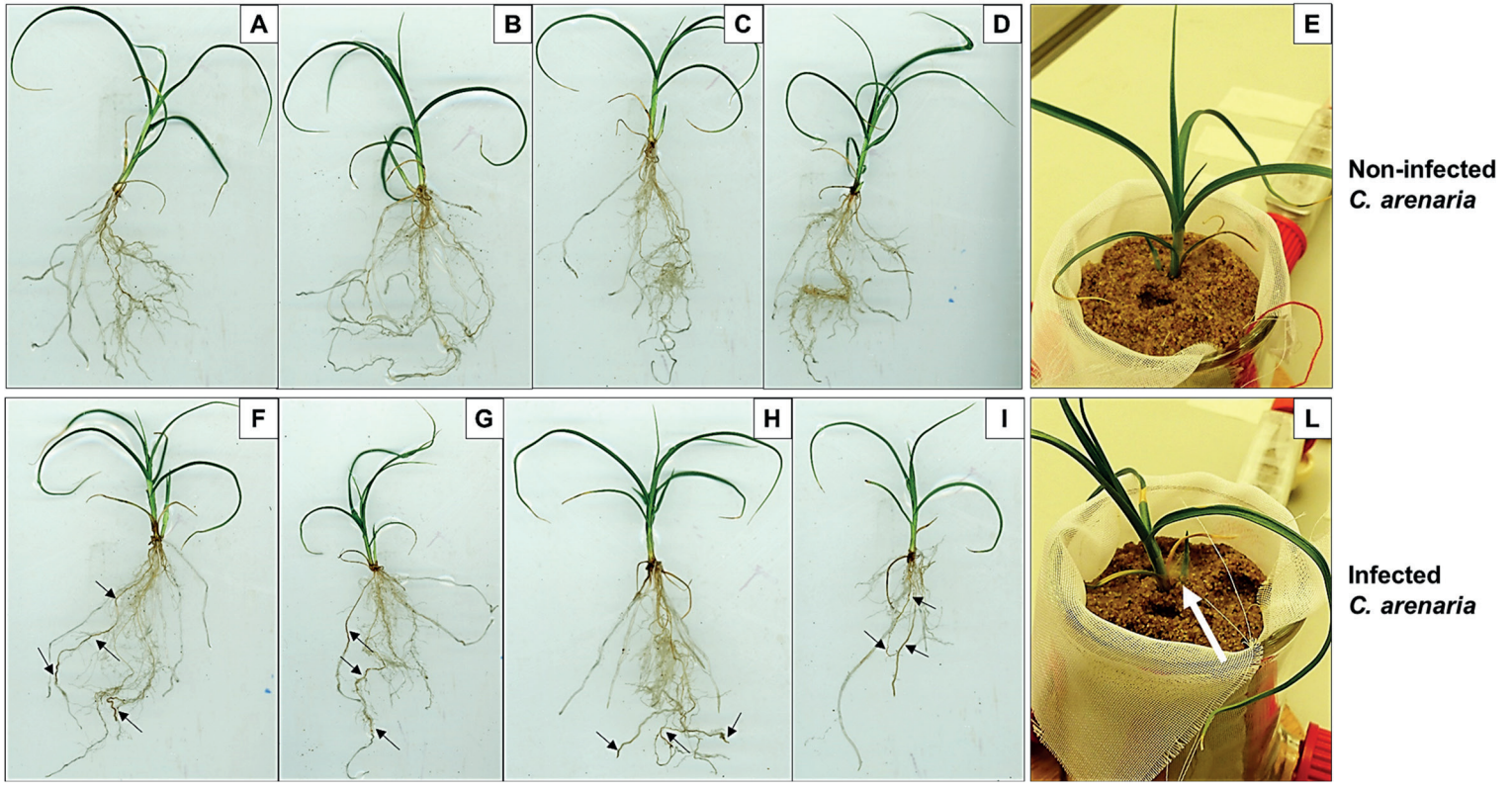

Figure S7.4. *Carex arenaria* incubated for 8 days in absence (A-E) or presence (F-L) of the soil-borne fungus *Fusarium culmorum* PV. Black arrows (F-I) indicate *F. culmorum*-infection areas at the plants roots. White arrow (L) shows fungal growth at the bottom of the plant shoot.

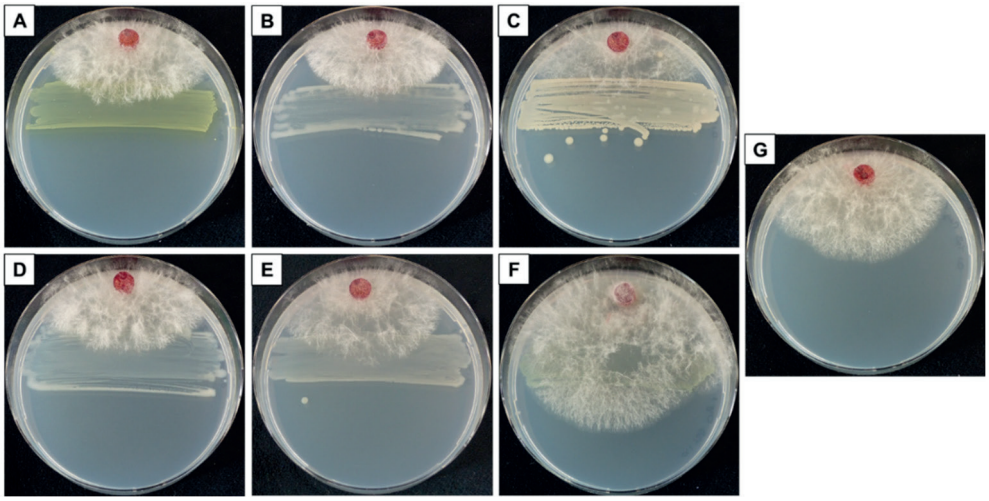

Figure S7.5. Direct interaction assay of *F. culmorum* PV with the rhizobacteria: (A) *Burkholderia* sp. AD024, (B) *Pseudomonas* sp. AD021, (C) *Paenibacillus* sp. AD087, (D) *Collimonas pratensis Ter91*, (E) *Janthinobacterium* sp. AD080 (F), *Dyella* sp. AD056 and (G) Control on 0.1 TSA.
Methodological details of the assay can be found in Supplementary Material 7.1.

Table S7.3. Number of migrated bacteria determined by qPCR in soil after 65 h of incubation. The bacteria in soil supplemented with or without artificial root exudates (ARE) were exposed to volatiles released from soil with or without *Carex arenaria* and/ or *Fusarium* culmorum PV and/ or. The average cell number and the ratio of migrated bacteria between the treatments and control are listed with the corresponding standard error (SE). Hash indicates significant difference ($p < 0.05$) between the treatments and the control. In terms of the calculated ratios, asterisks indicate significant difference ($p < 0.05$) between soil treatments without and with ARE.

Treatments		*Burkholderia sp.* AD024		*Dyella sp.* AD056		*Collimonas pratensis* Ter91		*Janthinobacterium sp.* AD080		*Paenibacillus sp.* AD087		*Pseudomonas sp.* AD021	
		Mean ± SE (Cells g^{-1} soil)	Treat/ Control ± SE	Mean ± SE (Cells g^{-1} soil)	Treat/ Control ± SE	Mean ± SE (Cells g^{-1} soil)	Treat/ Control ± SE	Mean ± SE (Cells g^{-1} soil)	Treat/ Control ± SE	Mean ± SE (Cells g^{-1} soil)	Treat/ Control ± SE	Mean ± SE (Cells g^{-1} soil)	Treat/ Control ± SE
No ARE	Control	$7.7x10^3 \pm 1.3x10^3$	-	$6.1x10^4 \pm 1.9x10^4$	-	$2.1x10^3 \pm 4.2x10^2$	-	$5.0x10^6 \pm 1.0x10^6$	-	$1.6x10^2 \pm 5.1x10^1$	-	$2.2x10^6 \pm 6.1x10^5$	-
	Carex	$2.5x10^4 \pm 7.7x10^{3\#}$	3.3 ± 0.4*	$1.3x10^5 \pm 4.5x10^{4\#}$	2.1 ± 0.3*	$3.2x10^3 \pm 5.7x10^{2\#}$	1.5 ± 0.1*	$9.2x10^6 \pm 1.8x10^{6\#}$	1.8 ± 0.1	$1.1x10^2 \pm 1.2x10^1$	0.7 ± 0.03	$5.8x10^6 \pm 1.5x10^{6\#}$	2.7 ± 0.2*
	Carex + F.*culmorum*	$1.4x10^4 \pm 3.2x10^{3\#}$	1.8 ± 0.1*	$1.5x10^5 \pm 2.7x10^{4\#}$	2.4 ± 0.2*	$2.5x10^3 \pm 2.9x10^2$	1.2 ± 0.05	$6.2x10^6 \pm 1.2x10^6$	1.2 ± 0.08	$1.3x10^2 \pm 3.2x10^1$	0.7 ± 0.02	$5.3x10^6 \pm 8.5x10^{6\#}$	2.4 ± 0.1*
	F. culmorum	$6.5x10^3 \pm 1.2x10^3$	0.8 ± 0.06*	$5.8x10^4 \pm 1.6x10^4$	1.0 ± 0.1	$1.8x10^3 \pm 1.7x10^2$	0.9 ± 0.03	$2.2x10^6 \pm 3.2x10^{5\#}$	0.5 ± 0.04	$2.0x10^3 \pm 7.7x10^1$	1.1 ± 0.1	$2.5x10^6 \pm 4.9x10^5$	1.2 ± 0.8*
+ ARE	Control	$7.4x10^6 \pm 2.5x10^6$	-	$2.2x10^5 \pm 2.6x10^4$	-	$8.8x10^4 \pm 1.5x10^4$	-	$3.3x10^8 \pm 1.3x10^8$	-	$1.4x10^5 \pm 3.4x10^4$	-	$2.5x10^8 \pm 3.3x10^7$	-
	Carex	$4.5x10^6 \pm 9.1x10^5$	0.6 ± 0.04	$2.2x10^5 \pm 2.6x10^4$	1.3 ± 0.07	$9.1x10^4 \pm 9.7x10^3$	1.0 ± 0.04	$1.5x10^9 \pm 1.5x10^{8\#}$	1.4 ± 0.05	$1.4x10^5 \pm 1.7x10^4$	1.0 ± 0.05	$3.0x10^9 \pm 4.7x10^7$	1.2 ± 0.07
	Carex + *F.culmorum*	$3.9x10^6 \pm 1.2x10^6$	0.5 ± 0.06	$1.9x10^5 \pm 1.8x10^4$	0.9 ± 0.08	$1.6x10^5 \pm 1.8x10^{4\#}$	1.9 ± 0.07	$2.1x10^9 \pm 1.8x10^{8\#}$	2.1 ± 0.06	$2.6x10^5 \pm 3.8x10^{4\#}$	1.9 ± 0.1	$3.8x10^8 \pm 3.5x10^{7\#}$	1.6 ± 0.05
	F. culmorum	$5.2x10^5 \pm 1.1x10^{5\#}$	0.07 ± 0.01	$2.9x10^5 \pm 4.5x10^{4\#}$	0.4 ± 0.04	$8.3x10^4 \pm 1.1x10^4$	0.9 ± 0.05	$9.5x10^8 \pm 9.8x10^7$	0.9 ± 0.04	$7.1x10^4 \pm 1.0x10^{4\#}$	0.5 ± 0.03	$2.1x10^8 \pm 4.4x10^7$	0.9 ± 0.07

Table S7.4. Volatile compounds produced the by fungal pathogen *Fusarium culmorum* PV in soil and by roots of *Carex arenaria* infected (Carex + F. culmorum) or non-infected (Carex) by *F. culmorum*. VOCs that could not be assigned with certainty to a known compound in the reference libraries remained unknown.

Treatment (total number)	Compound	Compound class	RI
Carex	Styrene	Aromatic compound	884
(6)	Acetophenone	Aromatic ketone	969
	N-tert-butylcyanoacetamide	Amide	1317
	Ethyl-1H-tetrazole-5-acetate	Ester	1533
	5-Ethyl-1,2,3,4-tetrahydro-naphthalene	Aromatic compound	1534
	2,4-diphenyl-4-Cyclopentene-1,3-dione	Ketone	2225
Carex +	1-Ethylcyclopropene	Alkene	570
F. culmorum	1,2-Dichloro-propane	Alkane	710
(17)	1-Chloro-2-nitropropane	Alkane	758
	1,2,3-Trimethylcyclohexane	Alkane	886
	Unknown	n.s.	958
	Benzofuran*	Aromatic ether	997
	Unknown	n.s.	1102
	2,6-Dimethyl-3,5,7-octatriene-2-ol	Alcohol	1127
	(Z)-Limonene oxide	Terpene	1127
	Tetrafluoroterephthalonitrile	Aromatic compound	1139
	1-(Pentafluorophenyl)benzene	Aromatic compound	1230
	7-Thioxo-1,7-dihydro-triazolo(4,3-b)-triazine	Sulfur compound	1486
	2,4-Dibromo-5-methoxybenzaldehyde	Aromatic aldehyde	1593
	1-(phenylmethylene)-1H-Indene	Aromatic compound	1863
	Isopropyl palmitate	Ester	2024
	2-Benzofuranylphenyl-methanone	Aromatic ether	2058
	Unknown	n.s.	2472
F. culmorum	Propanal*	Aldehyde	536
(40)	Isoprene	Alkene	542
	Acetaldoxime	Alcohol	555
	Unknown	n.s.	557
	Amylene hydrate*	Alcohol	650
	2-Ethyl-4-pentenal	Aldehyde	837
	2-Methyl-5-isopropylfuran	Aromatic ether	848
	3-Methyl-1-pentanol	Alcohol	867
	3-Heptanone	Ketone	879
	2-Ethyl-phenol	Aromatic compound	913
	Unknown	n.s.	919
	α-Pinene*	Terpene	930
	3-Methyl-isoxazol-5(4H)-one	Ketone	939
	Camphene*	Terpene	948
	3-Octanone*	Ketone	989

Table S7.4 continued.

Treatment (total number)	Compound	Compound class	RI
F. culmorum	2-Octanone	Ketone	994
	5-Formyl-4-nonene	Aldehyde	1019
	Isocineole	Terpene	1019
	o-Cymene	Aromatic compound	1028
	1,8-Cineole	Terpene	1036
	2-Ethyl-2,6-dimethyl-5,6-dihydro-2H-pyran	Ether	1037
	3-Isopropyl-5-methyl-hex-4-en-2-one	Ketone	1037
	2,2,4,6,6-Pentamethylheptane	Alkane	1044
	5-Nonanone	Ketone	1074
	α-Fenchone	Terpene	1088
	2-Nonanone	Ketone	1091
	Fenchol	Terpene	1115
	Unknown	n.s.	1130
	2,2,6,6-Tetramethyl-3,5-heptanedione	Ketone	1131
	2-Cyclohexylpropionaldehyde	Aldehyde	1151
	Endo-borneol	Terpene	1164
	1-Methylene-1H-indene	Aromatic compound	1174
	α-Terpineol	Terpene	1187
	3-Isobutyl-5-methyl-2-hexanone	Ketone	1194
	Unknown	n.s.	1201
	Benzothiazole*	Sulfur compound	1215
	Unknown	n.s.	1238
	L-α-Bornyl acetate	Terpene	1282
	6-Dodecanone	Ketone	1298
	Diphenyl ether	Ether	1405
Carex/ F. culmorum (1)	Dodecane	Alkane	1194
Carex/	2-Methyl-1H-pyrrole	Aromatic compound	561
Carex +	Dimethyl disulfide*	Sulfur compound	752
F. culmorum	3-Methylcyclopentyl acetate	Ester	894
(53)	3-Ethyl-2,5-dimethyl-hexane	Alkane	895
	Benzaldehyde	Aromatic aldehyde	960
	1-Ethyl-4-methyl-benzene	Aromatic compound	978
	Phenol*	Aromatic compound	981
	Benzonitrile*	Aromatic compound	985
	Unknown	n.s.	990
	Hexanoic acid	Carbonic acid	996
	Benzeneacetaldehyde	Aromatic aldehyde	1046
	γ-Caprolactone	Ester	1052
	Vinyl benzoate	Aromatic compound	1066
	5-Tert-butyl-5-methylbenzene-1,2-diol	Alcohol	1080

Table S7.4 continued.

Treatment (total number)	Compound	Compound class	RI
Carex/ Carex + F. culmorum	Ethaneperoxoic acid, 1-cyano-1-phenylbutyl ester	Ester	1146
	4-Cyclopropyl-pyrimidine	Aromatic compound	1191
	Valeric anhydride	Ester	1249
	Nonanoic acid*	Carbonic acid	1268
	1-Ethyl-1,2,3,4-tetrahydro-naphthalene	Aromatic compound	1330
	2-bromo-1-phenyl-1-Propanone	Aromatic ketone	1343
	γ-Nonalactone*	Ester	1363
	Neopentyl 2,2-dimethylbutanoate	Ester	1390
	Diphenylmethane	Aromatic compound	1433
	γ-Decalactone	Ester	1464
	2-Methoxyhydroquinone	Aromatic alcohol	1504
	Diphenyl sulfide	Sulfur compound	1577
	4-Phenyl-4-cyclopentene-1,3-dione	Alcohol	1581
	Benzophenone	Aromatic ketone	1622
	2,3-Dihydro-1,1,3-trimethyl-3-phenyl-1H-indene	Aromatic compound	1707
	Phenyl benzyl ketone	Ketone	1728
	9H-Fluoren-9-one	Ketone	1736
	Unknown	n.s.	1747
	3-Chloro-2-ethyl-5-(styryl)furan	Aromatic ether	1754
	1,2-Diphenoxyethane	Aromatic ether	1804
	Benzil*	Aromatic ketone	1812
	Hexadecahydro-benzo[de]anthracene	Aromatic compound	1816
	1-Dodecylbenzene	Aromatic compound	1834
	Diisobutyl phthalate	Aromatic compound	1859
	4-(Phenylvinyl)nicotinaldehyde	Aromatic aldehyde	1882
	3-Phenyl-2-benzofuran-1(3H)-one	Ester	1951
	2-(2-Pyridyl)quinoline	Aromatic compound	1984
	1,3-Epimanoyl oxide	Terpene	2040
	2-Naphthyl phenyl ether	Aromatic ether	2050
	3-Phenylcoumarin	Aromatic ketone	2099
	Pyrene	Aromatic compound	2150
	5-(2-Pyridyl)-6-phenyl-2-pyridinone	Aromatic ketone	2167
	m-Terphenyl	Aromatic compound	2173
	p-Terphenyl	Aromatic compound	2214
	2,5-Diphenyl-2,5-cyclohexadiene-1,4-dione	Ketone	2225
	2,6-Diphenylphenol	Aromatic compound	2251
	4-Phenyldibenzofuran	Aromatic ether	2322
	3-Methyl-3,4-dihydro-1H-2,3-benzothiazine 2,2-dioxide	Sulfur compound	2340
	2-[N,N-Bis(2-hydroxyethyl)aminomethyl]-7-methoxycoumarin	Aromatic ketone	2597

Table S7.4 continued.

Treatment (total number)	Compound	Compound class	RI
Carex+	2-Methylfuran	Aromatic compound	615
F. culmorum/	1,3-bis(1,1-dimethylethyl)-Benzene	Aromatic compound	1242
F. culmorum	Phenylbenzene	Aromatic compound	1383
(6)	4-Ethylisoquinoline	Aromatic alcohol	1476
	(+)-Acora-3,7(14)-diene	Terpene	1420
	(Z)-Muurola-3,5-diene	Terpene	1435
Carex/	Acetic acid ethyl ester	Ester	618
Carex+	2-Pentanone	Ketone	694
F. culmorum/	Phenylacetylene	Aromatic compound	869
F. culmorum	Hexanoic acid methyl ester	Ester	921
(10)	1-Dodecene	Alkene	1185
	Diethyl Phthalate	Aromatic compound	1576
	1-Pentadecyne	Alkyne	1608
	Dodecyl acrylate	Ester	1690
	Docosane	Alkane	2202
	Tricosane	Alkane	2298

n.s. not specified.
RI Linear retention Index of a 30 × 0.25 mm ID RXI-5MS column.
* Verified by co-injection with pure compound.

Table S7.5. Relative peak areas of diffused pure volatile compounds along different distances in soil. Data represent mean values (N=6) and standard errors (in %) of peak areas related to the area measured at distance D1 per compound. Different letters indicate significant difference ($p < 0.05$) between relative peak areas tested for each compound group (i.e. a-c compounds related to the VOCs blend of *Carex*, d-f compounds related to the blend of *F. culmorum*).

Compound	Distance D1 (0-0.5 cm)	Distance D2 (2.5-3 cm)	Distance D3 (5.5-6 cm)	Distance D4 (8.5-9 cm)	Distance D5 (11.5-12 cm)
α-Pinene	100.0	14.7 ± 6.4	22.9 ± 14.4	1.4 ± 0.6	0.7 ± 0.5^{f}
Amylene hydrate	100.0	21.3 ± 11.6	4.6 ± 2.4	0.0	0.0^{f}
Benzil	100.0	77.0 ± 18.1	35.5 ± 8.5	79.1 ± 24.6	54.5 ± 12.1^{b}
Benzofuran	100.0	46.7 ± 6.2	21.2 ± 7.9	7.3 ± 2.9	2.7 ± 1.4^{c}
Benzonitrile	100.0	9.5 ± 4.7	4.6 ± 2.6	5.0 ± 3.7	3.1 ± 2.3^{c}
Benzothiazole	100.0	24.7 ± 14.3	10.3 ± 7.3	42.5 ± 40.1	16.4 ± 14.4^{ef}
Camphene	100.0	30.2 ± 13.3	34.4 ± 19.8	22.7 ± 16.7	28.8 ± 21.4^{ef}
Dimethyl disulfide	100.0	99.8 ± 25.3	76.8 ± 21.4	92.6 ± 29.7	77.8 ± 22.4^{a}
γ-Nona-lactone	100.0	77.0 ± 8.1	69.6 ± 7.1	77.3 ± 17.1	81.6 ± 14.5^{a}
Nonanoic acid	100.0	70.4 ± 10.1	67.0 ± 13.0	72.9 ± 11.8	53.9 ± 7.2^{b}
3-Octanone	100.0	56.6 ± 7.3	37.7 ± 6.6	30.8 ± 9.2	39.0 ± 11.0^{de}
Phenol	100.0	63.0 ± 10.2	56.9 ± 16.0	75.5 ± 15.9	48.2 ± 9.1^{b}
Propanal	100.0	88.2 ± 11.7	88.7 ± 4.9	100.0 ± 12.9	105.6 ± 16.5^{d}

CHAPTER 8

General Discussion

Similar to plants, microbes produce a wide set of diverse volatile organic compounds (VOCs), small molecules with the ability to diffuse through air- and water-filled soil pores (Insam & Seewald 2010). It is increasingly recognized that those compounds can play important roles in microbe-microbe or microbe-plant interactions (Effmert *et al.* 2012; Van Dam *et al.* 2016; Schulz-Bohm *et al.* 2017c). However, our knowledge on the complexity and role of VOCs-mediated interactions belowground is still limited. The aim of this thesis was to reveal new insights into the ecological relevance of VOCs in microbial interactions and community dynamics belowground. In this regard, I studied various microbe-microbe as well as plant-microbe interactions (Figure 8.1). Main findings of this thesis are further discussed in this chapter.

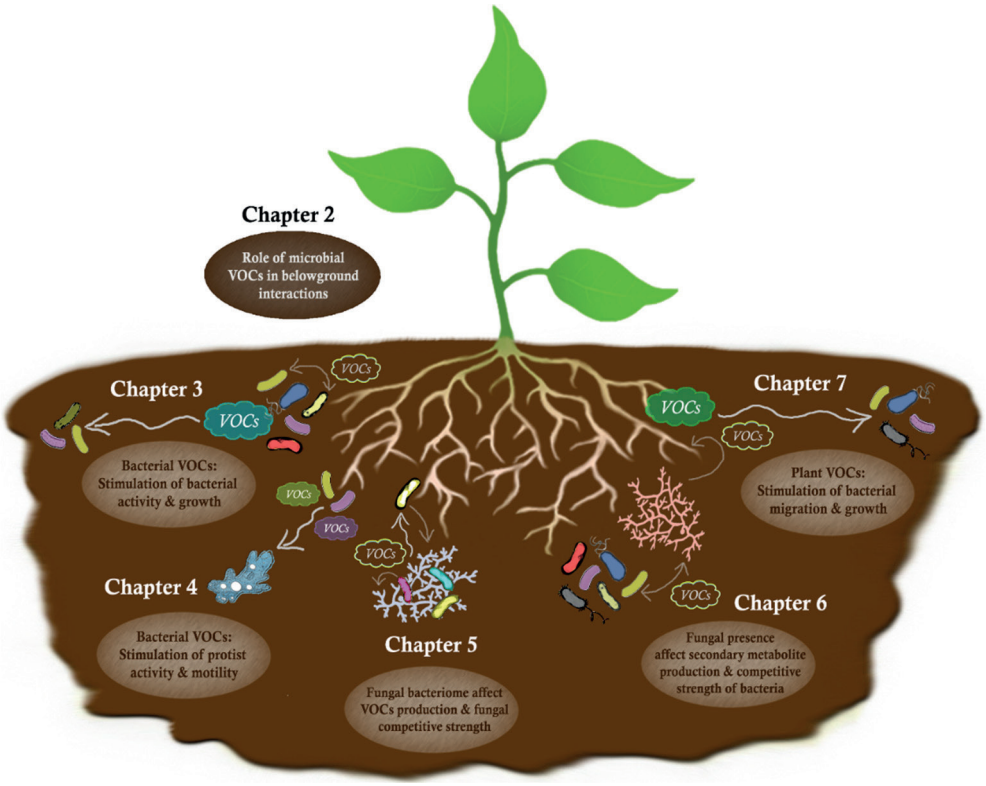

Figure 8.1. Summary of main findings of the thesis.

Factors Influencing the Composition and Biological Activity of VOCs

The composition of microbial as well as plant VOCs can be very dynamic and is affected by multiple abiotic and biotic factors (Insam & Seewald 2010; Peñuelas *et al.* 2014). For example, elevated soil temperatures can enhance the VOCs production while increase in soil moisture lead to a decrease in VOCs emission (Asensio *et al.* 2007). In Chapter 5, we observed that the saprotrophic fungus *Mucor hiemalis* emitted less VOCs when incubated on nutrient-poor water-agar compared to nutrient-rich potato dextrose agar. This is in line with previous studies showing that the production of VOCs is strongly dependent on available nutrient sources and that different nutrient sources can even lead to contrasting effects of VOCs-mediated interactions (Bruce *et al.* 2000; Blom et al. 2011; Garbeva et al. 2014b; Schmidt et al. 2016; Lazazzara *et al.* 2017). Likewise, the type of culture medium can affect the microbial VOCs production (Weise *et al.* 2012) and a different blend of VOCs can be emitted when bacteria are incubated in soil compared to agar (Garbeva et al. 2014b). Therefore, to study the ecological relevance of VOCs-mediated interactions belowground in Chapter 3, 6 and 7, we designed an experimental system that more closely mimics the *in situ* conditions in soil environments. Moreover, we studied interactions of soil microbes in a community context instead of the commonly used one-to-one species interactions.

In Chapter 3 and 6, we showed that interspecific interactions and shifts in the microbial community composition strongly affect the volatile blend released from soil. The relationship between community composition and VOCs production is in accordance with previous studies (Garbeva *et al.* 2014a; Hol *et al.* 2015; Tyc *et al.* 2015; Van Agtmaal *et al.* 2015; El Ariebi et al. 2016). In this regard, the presence of low-abundant species in the microbial community, such as observed for the *Paenibacillus* strain in Chapter 3, can significantly contribute to the emission of certain VOCs such as pyrazines with antifungal activity (Hol *et al.* 2015).

In Chapter 5, we revealed that a diverse bacterial community is associated with the saprotrophic fungus *Mucor hiemalis* and that those bacteria affect the secondary metabolite production of the fungus. While the importance of endobacteria on the fungal VOCs production in terms of aroma was previously shown for truffles (Splivallo *et al.* 2015; Vahdatzadeh *et al.* 2015) it was not known that saprotrophic fungi can harbor a diverse community of bacteria. We demonstrated that the *M. hiemalis*-associated bacteriome had a significant effect on the volatile blend and competitive strength of this fungus. For instance, shifts in the fungus-associated bacterial community led to changes in VOCs emission that stimulated growth and motility of bacterial competitors such as the fungus-feeding *Collimonas fungivorans* (Chapter 5).

Generally, changes in the VOCs profile by shifts in the microbial community composition and interspecific interactions can be explained by various factors. (1) Some VOCs are only produced under certain (stress) conditions such as competitive interactions (El Ariebi et al. 2016; Tyc *et al.* 2017a) or pathogenic infestation (Henriquez *et al.* 2012; Becker *et al.* 2014). For instance, in Chapter 6 we observed that during competitive interactions with the fungus *Fusarium culmorum* genes related to terpene synthesis were up-regulated by *Dyella sp*. and *Collimonas pratensis* and more terpenes were emitted. Furthermore, in Chapter 7 we have seen an increased emission of terpenes upon infection of *Carex arenaria* roots by *F. culmorum*. (2) Interspecific interactions can also silence the production of specific VOCs (Garbeva *et al.* 2014a; Tyc *et al.* 2015) and (3) VOCs emitted by one organism can be consumed or degraded by interacting partners. The latter can be linked to the detoxification of VOCs with antimicrobial activity (Cooney *et al.* 2001; Chapter 6). (4) The consumption of VOCs can result in a biotransformation of those compounds. For example, some fungi can convert acetoin to 2,3-butanediol isomers, active in plant growth promotion and induction of systemic resistance (Javidnia *et al.* 2016). Furthermore, several bacteria and fungi are able to oxidize and transform monoterpenes (Marmulla & Harder 2014).

In summary, besides the growth stage (e.g. Weikl *et al.* 2016), an important biotic factor affecting the blend of bioactive VOCs released in soil is the direct interplay between soil (micro-) organisms in the community. Moreover, the composition of the microbial community, with regard to who is there and interacting with whom, might be crucial concerning the emission of bioactive VOCs that are relevant for the belowground ecosystem functioning. In this respect, as shown for aboveground plant-plant, plant-insect or insect-insect interactions (e.g. D'Alessandro *et al.* 2009; Ninkovic 2010; Bruce & Pickett 2011) the right mix of VOCs could be a essential component for the biological activity in belowground interactions. Furthermore, similar to antibiotics (Raaijmakers & Mazzola 2012), it can be suggested that VOCs function in a concentration-dependent manner. For instance, it was shown that the fungal terpene 3-carene stimulated bacterial motility at low concentrations while high concentrations inhibited motility (Schmidt *et al.* 2016). On the other hand, the biological activity of VOCs can be enhanced by synergistic effects between different volatile compounds (De Vrieze *et al.* 2015; Mookherjee *et al.* 2017) or volatile and non-volatile compounds (Tyc *et al.* 2017a; Tyc *et al.* 2017b).

The Role of Volatile Mediated Interactions in Ecosystem Functioning Belowground

VOCs are suggested as long-distance messengers (Schulz-Bohm *et al.* 2017c). However, how far those compounds can travel in soil is not well known. In Chapter 7, we revealed that VOCs of diverse chemical classes including propanal, γ-nonalactone and dimethyl disulfide can diffuse over distances of about 12 cm in a soil matrix. However, the detectable amounts of some compounds such as amylene hydrate, benzofuran and benzothiazole decreased drastically with sampling distance. With the fact that VOCs probably act in a concentration-dependent manner, those compounds might be perceived differently by closely located soil organisms as compared to distant organisms. For example, in close proximity to the producer, VOCs such as benzofuran or benzothiazole could act in high concentrations as antimicrobial while further away from the producer those compounds may fulfil other functions such as stimulation of growth or motility. In addition, some VOCs may generally act as short-distance messenger while others facilitate interactions of soil organisms over long distance. For instance, Hiltpold and Turlings (2008) suggested that certain sesquiterpenes such as (E)-ß-caryophyllene emitted by plant roots could function as long-distance messenger.

Plants can release VOCs to attract beneficial soil organisms. For example, it is known that entomophatogenic nematodes can be attracted by plant VOCs such (E)-ß-caryophyllene released upon herbivory by root-feeding beetle larvae (Rasmann *et al.* 2005). In Chapter 7, we revealed for the first time that plant VOCs play also an important role in long-distance plant-microbe interactions, specifically in the attraction of bacteria from outside the rhizosphere. Moreover, by using a newly designed olfactometer system we demonstrated that bacteria with antifungal properties such as *Collimonas pratensis*, *Janthinobacterium sp*. and *Paenibacillus sp*. were attracted by VOCs released from infected roots of *C. arenaria* with the fungus *F. culmorum*.

The importance of VOCs as long-distance messengers in belowground microbe-microbe interactions was shown in Chapter 3 and 4. In Chapter 3, we demonstrated that VOCs released by a bacterial community with access to root exudates strongly stimulated the activity of starved bacteria in the surrounding nutrient-depleted bulk soil. This was correlated to an induction or, in some cases, even inhibition of growth of distant bacteria. Hence, VOCs produced by rhizosphere colonizing bacteria can strongly affect community dynamics of distant soil microbes. For instance, a recent study by Yuan *et al.* (2017) showed that bacterial VOCs could alter the composition of soil bacterial and fungal communities and could decrease the alpha-diversity of the soil microbial community. Moreover, in combination with the fact that plant root VOCs can also influence distant soil bacteria

(Chapter 7), one could argue that the rhizosphere effect is actually not restricted to the narrow zone around the roots as initially defined by Hiltner (1904) but that VOCs-mediated interactions can expand the rhizosphere effect further than the few millimeters around the roots (Dotaniya & Meena 2015). In this respect, VOCs released by plant roots and rhizosphere inhabiting microbes can affect important processes of the soil nitrogen cycle (Bending & Lincoln 2000; Insam & Seewald 2010; Yuan *et al.* 2017). For example, VOCs of the rhizobacterium *Bacillus amyloliquefaciens* were shown to increase the number of ammonium-oxidizing soil bacteria while the numbers of nitrogen fixing bacteria and denitrifiers was decreased (Yuan *et al.* 2017). Furthermore, it was shown that monoterpenes released by plant roots affected nitrogen transformation and nitrification in soil (Ward *et al.* 1997; Paavolainen *et al.* 1998; Smolander *et al.* 2006).

Besides as info-chemicals, biogenic VOCs can serve as carbon source for soil microbes (Kleinheinz *et al.* 1999; Owen *et al.* 2007; Ramirez *et al.* 2009). This might explain the increase in numbers of nutrient-limited bacteria by exposure to microbial and plant VOCs observed in Chapter 3 and 7. Hence, VOCs-mediated microbial and plant interactions also play a role in the soil carbon cycle.

Important players contributing to carbon allocation and, accordingly, to soil nutrient-cycles are protists (Geisen 2016). Protists are key predators of bacteria and can shape bacterial communities by selective feeding (Bonkowski & Brandt 2002; Rosenberg *et al.* 2009; Glücksman *et al.* 2010). Since reaching the prey can be very costly (Jousset 2012), bacterial VOCs could give early information on suitable prey. In Chapter 4, we revealed for the first time that VOCs released by various bacteria such as terpenes can stimulate protist activity. This was mostly correlated to an increase in activity when protists could directly feed on bacteria. In some cases, VOCs of bacteria decreased protist activity. This suggests that VOCs can be of key importance in bacteria-protist predator-prey-interactions and highlights the involvement of VOCs-mediated chemical communications in the regulation of soil food webs (DeAngelis 2016). Another example adding to the fact that soil food webs can be connected by VOCs-mediated communication is the beneficial effect of bacterial VOCs on the development of mycorrhizal fungi (Carpenter-Boggs *et al.* 1995; Effmert *et al.* 2012). Furthermore, in Chapter 5 we revealed that changes in the bacterial community associated to the fungal host can lead to shifts in the VOCs profile that resulted in an attraction instead of previous repellence of fungus-feeding (i.e. mycophageous [Leveau *et al.* 2010]) bacteria.

Besides in nutrient-cycles and soil food webs, microbial VOCs do also play a role in other relevant soil processes as summarized in Chapter 2. For instance, VOCs-mediated interactions between microbes can contribute to the suppression of soil-borne plant

diseases (Schulz-Bohm *et al.* 2017c) and can be involved in soil fungistasis (Garbeva *et al.* 2011a). Furthermore, microbial VOCs can enhance plant growth and improve nutrient access of plants (Zhang *et al.* 2009; Bailly & Weisskopf 2012; Meldau *et al.* 2013; Bitas *et al.* 2013; Peñuelas *et al.* 2014; Li *et al.* 2016; Van Dam *et al.* 2016; Riedlmeier *et al.* 2017).

Several studies have indicated that microbial VOCs can interfere with plant systemic resistance (Van Dam *et al.* 2016). For instance, Song and Ryu (2013) showed that the bacterial VOCs can induce systemic acquired resistance in cucumber that lead to resistance against the bacterial angular leaf spot pathogen and leaf-sucking aphids as well as induced the emission of green leaf volatiles recruiting the natural enemy of aphids. Cordovez *et al.* (2017) demonstrated that VOCs of the plant pathogen *Rhizoctonia solani* induced systemic resistance in *Arabidopsis thaliana* related to a decrease in resistance against aphids. Hence, VOCs-mediated interactions in soil do not only play important roles in ecosystem functioning belowground but can also affect aboveground interactions.

In conclusion, VOCs-mediated chemical communication can be very important for belowground ecosystem functioning. In this context, terpenes as one of the largest and most diverse class of metabolites that are produced by all kingdom of live including bacteria, fungi and plants (this thesis; Yamada *et al.* 2015; Chen *et al.* 2016) seem to play important roles in many soil processes (Chapter 2). For instance, terpene-mediated interactions can contribute to the suppression of soil-borne plant diseases (Song *et al.* 2015b; Chapter 6), stimulation of plant growth (Minerdi *et al.* 2011), soil nutrient cycles (Kleinheinz *et al.* 1999; Owen *et al.* 2007; Chapter 3), or the connection of soil food webs (Chapter 4). Furthermore, as several terpenes are commonly produced by different soil organisms, those compounds might function as "lingua franca" for intra- and inter-kingdom interactions belowground (Chapter 2).

Applications of Microbial and Plant Volatiles

Microbial VOCs can be quite effective against soil-borne plant diseases, even at very low concentrations (De Vrieze *et al.* 2015; Schulz-Bohm *et al.* 2017c). Hence, they are proposed as ecofriendly alternative compared to the commonly used hazardous pesticides in crop protection (Weisskopf 2013; Kanchiswamy *et al.* 2015b; Schalchli *et al.* 2016; Bailly & Weisskopf 2017). However, the activity of VOCs can strongly vary between lab and field conditions and because of high evaporation rates the application into open field can be challenging (Song & Ryu 2013; Kanchiswamy *et al.* 2015a). So far, experiences with the application of VOCs in the open field are limited. View studies showed that drench application of the bacterial VOCs (2,3)-butanediol, 3-pentanol and 2-butanone in the open

field can be very successful in the reduction of infection symptoms by various crop plants (Cortes-Barco *et al.* 2010a; Cortes-Barco *et al.* 2010b; Song & Ryu 2013). In Chapter 3, 4 and 7, we have seen that microbial or plant VOCs can activate and even attract distant microorganisms that could be beneficial for plant protection or growth. VOCs stimulating microbial activity could be applied as potential tool to enhance the effectiveness of biocontrol strains in the open field. A group of soil microbes that is often overlooked concerning biocontrol and the management of the rhizosphere microbiome are protists. Application of protists to plant rhizosphere can enhance the abundance and activity of bacteria with plant-beneficial traits including the production of toxic secondary metabolite or growth promoting hormones (Jousset 2017; Weidner *et al.* 2017). Hence, a stimulation of protist activity by exposure to certain microbial VOCs such as terpenes (Chapter 4) could enhance the effectiveness of protists in crop protection and growth promotion.

Besides in agriculture, microbial VOCs are also of interest in medicine and food production. They can be applied to monitor and counteract contaminations or infections of pathogenic microbes (Sankaran *et al.* 2011; Morath *et al.* 2012; Sethi *et al.* 2013; Ahmed *et al.* 2017) as well as for the flavor production (Deetae *et al.* 2007, Hadar & Dosoretz 1991). It is also proposed to use VOCs for the early detection of plant diseases (Laothawornkitkul *et al.* 2008).

In addition to biotechnological applications, VOCs can be of interest for basic scientific studies. For example VOCs can be used for genotyping of plants and microbes (Müller *et al.* 2013; Cordovez *et al.* 2015; Niederbacher *et al.* 2015) or as indicator for shifts of microbial community compositions as well as to compare various microbial communities (Mc Neal & Herbert 2009, Chapter 3 and 6).

A novel method to isolate fungus-associated bacteria was applied in Chapter 5. We used a mix of three VOCs (dimethyldisulfide, dimethyltrisulfide and benzonitrile) of known antifungal activity (Garbeva *et al.* 2014b) to successfully inhibit fungal growth and at the same time to stimulate the growth of fungus-associated bacteria. As biogenic VOCs can significantly increase the activity of soil bacteria and serve as carbon source or growth elicitor (Chapter 3 and 7), they can be a suitable tool for the isolation of so far unculturable microbes from soil.

Outlook for Future Research in the Field of VOCs Mediated Interactions

The findings of this thesis greatly contributed to our current knowledge on the ecological role of VOCs-mediated interactions belowground. However, there are still many

open questions. In the following paragraphs, some of those questions will be pointed out and given as suggestions for future research directions.

As discussed above, an important biotic component affecting the composition of VOCs are interspecific interactions. Since soil microbes usually occur in diverse communities, the ecological relevance of results obtained by studies on VOCs-mediated one-to-one species interactions remains to be questionable. Furthermore, to add on the knowledge gained in this thesis (specifically in **Chapter 7** and **4**) it would be interesting to test if and how the root-associated microbial community affect the attraction of (beneficial) microorganisms outside the rhizosphere and (2) if protists are equally attracted by VOCs of bacterial prey when they occur in a community.

In terms of the complexity and the role of VOCs produced by soil microorganisms, hardly anything is known about archaea and protists. Recently Chen *et al.* (2016) revealed that protists such as *Dictyostelium discoideum* produce terpenes, which might be involved in defense mechanisms, for example, to repel nematodes. However, since protists similar as fungi (**Chapter 5**) are probably never alone, the question arises if and in which way associated bacteria would be involved in the production of VOCs such as terpenes by protists.

To test the diffusion ability of various VOCs we used only one soil moisture level in **Chapter 7**. However, since soil moisture and soil type can influence the diffusion ability of VOCs (Hiltpold & Turlings 2008), it remains to be tested how relevant VOCs are as communication tool under different soil moisture levels.

Exposure to microbial VOCs can lead to global transcriptomic changes of soil microbes (Yung et al., 2015; Schmidt *et al.* 2017). However, as indicated in **Chapter 2** the perception of VOCs is largely unknown. Depending on the chemical character, VOCs could simply interfere with membrane structure (Maffei *et al.* 2001; Giorgio *et al.* 2015) to elicit downstream responses in the cell or bind to specific receptors of unknown properties. Transcriptomic analysis in combination with the construction of "deaf receiver" mutants might be a suitable approach to reveal perception mechanisms of VOCs.

Generally, microbes and plants produce a very diverse blend of VOCs that makes the search for potential receptors or perception mechanisms even more challenging (Werner *et al.* 2016). In this thesis, we tried to identify potential VOCs involved in specific microbe-microbe or plant-microbe interactions. However, a considerably amount of detected VOCs by GC-MS could not be assigned to known compounds and remained unknown. Thus, to find novel bioactive volatile compounds for potential biotechnological applications a close cooperation between various scientific disciplines is needed.

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SUMMARY

Soil microbes, similar as plants, produce a large set of diverse volatile organic compounds (VOCs), small molecules that can diffuse through air- and water-filled soil pores. It is increasingly recognized that these compounds can play important roles in various intra- and inter-kingdom interactions of physically separated soil (micro-) organisms (Chapter 2). However, our understanding of the complexity of VOCs-mediated interactions belowground is still scarce. The main aim of this thesis was to reveal novel insights into the relevance of VOCs in microbial interactions and community dynamics belowground. In this context, interactions between bacteria-bacteria, bacteria-fungi, bacteria-protists as well as plant-bacteria were examined.

Many studies on the role of VOCs in interactions of soil microorganisms used artificial conditions. As a way forward, a soil model system that more closely reflects the *in situ* conditions in the heterogeneous soil environment along the rhizosphere was designed to study VOCs-mediated interactions between soil bacteria (Chapter 3). It was revealed that microbial interactions and shifts in the microbial community composition had a strong effect on the volatile emission. Moreover, volatiles produced by interactions of bacteria with access to root-exudates strongly stimulated the activity of more distantly located starved bacteria. This was correlated with growth stimulation and, in some cases, growth reduction of bacteria exposed to those volatiles. Hence, volatiles produced during microbial interactions in the rhizosphere can have a significant long distance effect on microorganisms in the surrounding, nutrient-depleted bulk soil.

Bacterial VOCs could also function as signal molecules for other soil microbes such as protists. Protists are major predators of bacteria but reaching the prey can be very costly. Thus, VOCs released by bacteria could give early information on suitable prey located in distant soil pores. By testing the effect of bacterial VOCs on the performance of soil protists (Chapter 4), it was revealed that most bacteria emitting VOCs affected the activity of protists. However, the response of protists to bacterial VOCs was strongly dependent on the identity of the interacting partner. The stimulation of protist activity by bacterial VOCs was often correlated with the feeding response in direct interactions. This suggests that bacterial VOCs can serve as signals for protists to sense suitable prey. Furthermore, it was demonstrated that terpenes as an important group of volatile compounds produced by many bacteria are involved in protist-bacterial predator-prey interactions.

Besides protists, also fungi are abundant in the rhizosphere and can affect microbial community dynamics. Mutualistic and competitive interactions between bacteria and saprotrophic fungi were studied (Chapter 5 and 6). In case of the saprotrophic fungus *Mucor hiemalis*, the impact of fungus-associated bacteria on fungal fitness and behavior was analyzed (Chapter 5). Broad-spectrum antibiotics were initially applied to remove bacteria associated to *M. hiemalis*. However, instead of eliminating or reducing the amount of

bacteria the composition of the bacterial community was changed by the antibiotic treatment. The shifts in the composition of the fungus-associated bacterial community resulted in a significant reduction of fungal growth as well as changes in the morphology and secondary metabolite production, including VOCs. In this regard, VOCs released by the antibiotic-treated *M. hiemalis* stimulated growth and motility of potential antifungal bacteria such as the mycophagous *Collimonas fungivorans*. This shows that the fungus-associated bacteria can have an important role for the lifestyle and fitness of the fungal host.

In Chapter 6, the aim was to reveal bacterial competitive strategies in fungal presence. Soil microcosms containing a synthetic bacterial community in presence or absence of the fungus *Fusarium culmorum* were set up and quantitative PCR in combination with transcriptomics and volatilomics was performed to link changes in bacterial abundance to potential mechanisms. It was observed that the abundance of *Collimonas pratensis* and *Dyella sp*. significantly increased in fungal presence while the abundance of the other bacteria decreased. This was correlated with the total number of up-regulated and down-regulated genes per bacterium in fungal presence including genes related to secondary metabolite production (e.g. terpenes) and defense mechanisms (e.g. aromatic compound degradation). Additionally, the volatile blend produced by the bacterial community in fungal presence was characterized by an increase of terpenes and decrease of aromatic compounds as compared to bacterial growth in fungal absence. Generally, the increase in competitive strength of the bacteria in fungal presence was linked to the acquisition of fungus-derived-nutrients (i.e. mycophagy) in combination with the induction of secondary metabolite production and defense mechanisms.

In addition to microbe-microbe interactions, also VOCs released by plant roots can influence microbial community dynamics belowground. However, whether VOCs released by roots can attract (beneficial) soil microbes was not known. An olfactometer system was designed to test the attraction of soil bacteria to VOCs emitted by roots of *Carex arenaria* (sand-sedge). Furthermore, it was investigated how fungal infestation of *C. arenaria* roots by *Fusarium culmorum* affects the emission of VOCs and attraction of distant bacteria belowground (Chapter 7). It was revealed that the migration of distant bacteria in soil towards roots can be stimulated by plant VOCs. Upon fungal infection, the blend of root VOCs changed and specific bacteria with antifungal properties were attracted. Tests with various pure VOCs showed that these compounds can diffuse over long distance but with different diffusion abilities. Hence, plant VOCs can play important roles in long-distance plant-microbe-interactions belowground.

Overall, this thesis provides novel information on the ecological importance of VOCs in belowground microbe-microbe and plant-microbe interactions and strongly contributes to our understanding of VOCs-mediated interactions within the complex soil system.

SAMENVATTING & ZUSAMMENFASSUNG

SAMENVATTING

Micro-organismen in de bodem produceren, net als planten, een breed scala aan vluchtige organische stoffen (VOCs); kleine moleculen die zich verspreiden via lucht- en watergevulde poriën in de bodem. Het is bekend dat deze stoffen kunnen een belangrijke rol spelen in interacties binnen en tussen verschillende taxonomische rijken. Zoals bijvoorbeeld tussen bacteriën onderling en tussen bacteriën en schimmels. Deze interacties kunnen ook plaatsvinden wanneer de organismen ruimtelijk van elkaar gescheiden zijn (Hoofdstuk 2). Niettemin is nog steeds weinig bekend over de complexiteit van interacties tussen bodemorganismen via VOCs. In deze context zijn interacties tussen bacteriën, tussen bacteriën en schimmels, tussen bacteriën en protisten en tussen planten en bacteriën onderzocht.

In veel studies over de rol van VOCs in interacties tussen bodemmicro-organismen worden kunstmatig condities gebruikt. Al een stap vooruit is hier een modelsysteem gebruikt dat de *in situ* bodemcondities van het heterogene bodemmilieu rondom de rhizosfeer nabootst. Dit system is gebruikt om VOC-gestuurde interacties tussen bodembacteriën te bestuderen (Hoofdstuk 3). Hieruit bleek dat microbiële interacties en veranderingen in de compositie van de microbiële gemeenschap een groot effect hadden op de emissie van VOCs. Daarnaast bleek dat VOCs geproduceerd door interacties tussen bacteriën met toegang tot wortelexudaten de activiteit van uitgehongerde bacteriën in de distantie stimuleert. Dit was gecorreleerd met groeistimulatie en, in sommige gevallen, -reductie van bacteriën die aan deze VOCs werden blootgesteld. VOCs geproduceerd in de rhizosfeer kunnen daarom een significant langeafstandseffect op micro-organismen in de omgeving hebben als de bodem zelf is uitgeput qua voedingsstoffen. VOCs afgegeven door bacteriën kunnen daarom vroegtijdige informatie geven over een prooi die zich in bodemporiën de buurt bevindt.

Door het testen van het effect van bacteriële VOCs op de performance van bodemprotisten (Hoofdstuk 4) werd duidelijk dat de meeste bacteriën een effect hebben op bodemprotisten door het uitstoten van VOCs. De respons van protisten op de VOCs van bacteriën was niettemin afhankelijk van de partner waarmee de bacterie of protist interacteerde. De stimulatie van protistactiviteit door VOCs van bacteriën was vaak gecorreleerd met de response in directe voedingsinteracties. Dit suggereert dat VOCs van bacteriën gebruikt kunnen worden als signaal voor protisten om een geschikte prooi op te merken. Verder kon gedemonstreerd worden dat terpenen, een belangrijke groep van

VOCs geproduceerd door veel verschillende bacteriën, een rol spelen in protist-bacterie predator-prooi-interacties.

Naast protisten zijn ook schimmels abundant in de rhizosfeer, en dat kan een effect hebben op de dynamiek in de microbiële gemeenschap. Symbiotische en competitieve interacties tussen bacteriën en saprofytschimmels zijn bestudeerd (Hoofdstuk 5 en 6). Hierbij werd de schimmel *Mucor hiemalis* gebruikt om de impact van schimmelgerelateerde bacteriën op de performance van de schimmel te analyseren. Antibiotica met een breed spectrum waren hier initieel toegepast om bacteriën te verwijderen die geassocieerd waren aan *M. hiemalis*. Het bleek toen dat de antibiotica niet zorgde voor een reductie van het aantal bacteriën, maar voor een andere compositie. De verandering in de compositie van de schimmelgerelateerde bacteriën resulteerde in een significante reductie van de groei van de schimmel. Daarnaast veranderde het de morfologie en de productie van secondaire metabolieten op de schimmel, inclusief VOCs. In navolging hiervan zorgden de VOCs uitgestoten door de met antibiotica behandelde *M. hiemalis* voor een gestimuleerde groei en beweeglijkheid van antischimmel bacteriën zoals de schimmeletende *Collimonas fungivorans*. Dit demonstreert dat schimmelgerelateerde bacteriën een belangrijke rol kunnen hebben voor de lifestyle en fitness van de host.

In Hoofdstuk 6 was het doel om de competitieve strategieën van bacteriën in de aanwezigheid van schimmels te onderzoeken. In de proefopzet werden bodemorganismen met een synthetische bacteriële gemeenschap in de aanwezigheid en afwezigheid van de schimmel *Fusarium culmorum* gebruikt. Vervolgens werd kwantitatieve PCR in combinatie met 'transcriptomics' en 'volatilomics' gebruik om veranderingen in de bacteriële abundantie te linken aan potentiële mechanismen. Hier werd ontdekt dat de abundantie van *Collimonas pratentis* en *Dyella sp.* significant toenamen onder de aanwezigheid van schimmels, terwijl de abundantie van de andere bacteriën afnam. Dit was gecorreleerd met het totale aantal van aangezette en uitgezette genen per bacterie onder de aanwezigheid van schimmels, inclusief genen gerelateerd aan de productie van secondaire metabolieten (zoals terpenen) en verdedigingsmechanismen (zoals de degradatie van aromatische stoffen). Daarnaast bleek dat het mengsel van VOCs geproduceerd door de bacteriële gemeenschap in de aanwezigheid van schimmels veranderde. De terpenen namen toe en de aromatische stoffen namen af onder de aanwezigheid van schimmels, in vergelijking met de afwezigheid van schimmels. In zijn algemeenheid was een toename in de competitieve sterkte van bacteriën onder de aanwezigheid van schimmels gerelateerd aan de acquisitie van schimmelafgeleide nutriënten, in combinatie met de inductie van productie van secondaire metabolieten en verdedigingsmechanismen.

Naast interacties tussen microbiële organismen, kunnen VOCs ook via planten, die VOCs afscheiden uit de wortels, een invloed hebben op de microbiële gemeenschap en de dynamiek onder de grond. Het is echter nog niet bekend of VOCs afgegeven door wortels een attractief effect kunne hebben op (nuttige) bodemorganismen. Een systeem met een olfactometer was gebruikt om te kijken of bodembacteriën werden aangetrokken door VOCs, afgegeven door de wortels van de Zandzegge (*Carex arenaria*). Daarnaast is bekeken hoe door schimmels van *Fusarium culmorum* aangetaste wortels van de Zandzegge een invloed heeft op de afgifte van VOCs en de aantrekking of afstoting van bacteriën ondergronds (Hoofdstuk 7). Hier werd ontdekt dat de beweging van verder weggelegen bacteriën richting de wortels gestimuleerd kan worden door planten VOCs. Het mengsel van VOCs uit de wortels verandert na een infectie door schimmels. Dit hing samen met de aantrekking van specifieke bacteriën met een antischimmelwerking. Tests met verschillende pure VOCs lieten zien dat deze stoffen zich kunnen verspreiden over lange afstanden, maar de mate waarin ze zich verspreiding verschilt van stof tot stof. Planten VOCs kunnen dus een belangrijke rol spleen in de interacties tussen planten en micro-organismen over een langere afstand ondergronds.

Overall geeft dit proefschrift nieuwe informatie over de ecologische belangrijkheid van VOCs in ondergronds interacties tussen microben en tussen microben en planten. Het draagt daardoor bij aan de huidige kennis over VOC-gestuurde interacties in het complex bodem system.

Zusammenfassung

Mikroorganismen produzieren, ähnlich wie Pflanzen, eine Fülle von flüchtigen organischen Substanzen (volatile organic compounds; VOCs). Das sind kleine Moleküle, welche durch luft- und wassergefüllte Bodenporen diffundieren können. Studien haben im Laufe der letzten Jahrzehnte gezeigt, dass solche flüchtigen Substanzen eine wichtige Rolle in verschiedensten Interaktionen von Bodenorganismen wie Bakterien, Pilzen und Pflanzen spielen können, die im Boden getrennt voneinander leben (Kapitel 2). Allerdings wissen wir trotz voranschreitender Forschung immer noch sehr wenig auf diesem Gebiet. Ziel dieser Doktorarbeit ist es daher, neue Erkenntnisse über die ökologische Bedeutsamkeit von VOCs in mikrobiellen Interaktionen und für die Dynamik von mikrobiellen Gemeinschaftsstrukturen im Boden zu gewinnen. Dabei wurden Interaktionen zwischen verschiedenen Bakterien, Bakterien und Pilzen, Bakterien und Protisten als auch Pflanzen und Bakterien betrachtet.

Viele Arbeiten zur Funktion von VOCs in Interaktionen von Bodenmikroorganismen beruhen auf künstlichen Bedingungen. Deshalb ist ein Bodenmodell entwickelt worden, welches näher die tatsächlichen Gegebenheiten im Boden widerspiegelt. Mit diesem Modell sind über VOCs-bedingte Interaktionen zwischen Bodenbakterien untersucht worden (Kapitel 3). Die Versuchsergebnisse haben ergeben, dass Wechselwirkungen zwischen den Bakterien als auch eine andere Zusammensetzung der mikrobiellen Gemeinschaft einen großen Einfluss auf die Freisetzung von VOCs im Boden haben. Darüberhinaus konnte aufgezeigt werden, dass VOCs, welche durch Wechselwirkung von Bakterien mit Zugang zu Wurzelexudaten gebildet wurden, die Aktivität von nährstoff-limitierten räumlich getrennten Bakterien stark stimulieren können. Eine Erhöhung der Aktivität der Bakterien korrelierte mit einer Verstärkung des Zellwachstums oder, in machen Fällen, mit einer Inhibierung des Wachstums. Dies weist darauf hin, dass durch mikrobielle Interaktionen im Wurzelbereich freigesetzte VOCs Mikroorganismen, die im umgebenden nährstoff-limitierten Boden leben, beträchtlich beeinflussen können.

Neben Bakterien können bakterielle VOCs auch andere Bodenmikroorganismen, wie zum Beispiel Protisten, beeinflussen. Protisten sind eine Gruppe von nichtverwanden eukaryotischen Einzellern und sind wichtige Fressfeinde von Bakterien im Boden. Das Erreichen von geeigneter Beute im unterirdischen Labyrinth kann sehr kostenaufwendig für Protisten sein. Daher könnten VOCs, freigesetzt von Bakterien, als mögliches Signal zur Früherkennung von geeigneter Beute in weiter entfernten Bodenporen dienen. Um dies zu testen, sind verschiedene Protisten mit VOCs von unterschiedlichen Bakterien behandelt

worden (Kapitel 4). Die Ergebnisse belegen, dass die meisten Bakterien die Aktivität von Protisten mittels ihrer VOCs beeinflussen können, wobei eine positive oder negative Reaktion der Protisten auf die bakteriellen VOCs stark vom Interaktionspartner abhängig ist. Eine Stimulation der Aktivität von Protisten durch bakterielle VOCs hat oft mit dem Verhalten der Protisten korreliert, wenn diese direkt mit dem jeweiligen Bakterium in Kontakt standen und diese fressen konnten. Dies deutet daraufhin, dass bakterielle VOCs, wie zum Bespiel Terpene, in der Tat als mögliche Signale zur Erkennung von geeigneter Beute dienen können und somit eine wichtige Rolle in Prostisten-Bakterien-Räuber-Beute-Beziehungen spielen.

Außer Protisten leben im Wurzelbereich auch sehr viele verschiedene Pilze, deren Anwesenheit auch mikrobielle Gemeinschaftsstrukturen beeinflussen können. Insofern sind im Laufe der Doktorarbeit mutualistische (d.h. beiderseits positive) als auch kompetitive Wechselwirkungen zwischen Bakterien und Pilzen betrachtet worden. Im Falle des von toter organischer Substanz lebenden (saprotrophen) Pilzes *Mucor hiemalis* wurde der Einfluss von im Pilz lebenden Bakterien auf die Fitness und das Verhalten des Pilzes untersucht (Kapitel 5). Um bakterielle Pilzbewohner abzutöten, sind Antibiotika eingesetzt worden, wobei statt der Verringerung der Bakterienanzahl die Antibiotikabehandlung zur Veränderung der im Pilz lebenden Bakteriengemeinschaft beitrug. Dies hat wiederum Auswirkung auf den Pilz beziehungsweise sein Wachstum, Aussehen und die Produktion von sekundären Metaboliten, wie zum Beispiel VOCs. Unter anderem wurden VOCs vom mit Antibiotika behandelten Pilz freigesetzt, welche das Wachstum und die Bewegung von pilzinhibierenden Bakterien, wie zum Beispiel pilzfressenden Collimonaden, anregten. Dies zeigt, dass bakterielle Pilzbewohner eine wichtige Funktion für das Verhalten und die Fitness des Pilzes erfüllen können.

Konkurrierende Wechselwirkungen zwischen Bakterien und Pilz und die daraus resultierenden bakteriellen Strategien wurden am Modell von *Fusarium culmorum* und einer bakteriellen Gemeinschaft in Bodenmikrokosmen untersucht. In Gegenwart des Pilzes *F. culmorum* ist es zur Zunahme der Bakterienspezien *Collimonas pratensis* und *Dyella sp.* gekommen, während die Anzahl aller anderen Bakterienspezien in der bakteriellen Gemeinschaft abnahm. Dies hat mit der Anzahl von hoch- oder herunterregulierten Genen pro Bakterium in Gegenwart des Pilzes korreliert. Dazu gehören Gene, die zur Herstellung von sekundären Metaboliten (z.B. Terpene) und Abwehrmechanismen (z.B. Abbau von giftigen aromatischen Substanzen) benötigt werden. Eine Analyse der Zusammensetzung von freigesetzten VOCs hat zusätzlich ergeben, dass mehr Terpene und weniger aromatische Substanzen in Gegenwart des Pilzes durch die bakterielle Gemeinschaft gebildet wurden. Insgesamt konnte die verstärkte Konkurrenzfähikeit von *Collimonas*

pratensis und *Dyella sp.* in Gegenwart des Pilzes mit der Fähigkeit, sich vom Pilz zu ernähren, und einer Stimulation von Abwehrmechanismen als auch der Bildung von sekundären (antimikrobiellen) Metaboliten verbunden werden.

Zusätzlich zu VOCs-bedingten Wechselwirkungen zwischen Mikroorganismen können auch von Pflanzen gebildete flüchtige Substanzen mikrobielle Gemeinschaftsstrukturen im Boden beeinflussen. Eine positive Wirkung von pflanzlichen VOCs, wie das Anlocken von (nützlichen) Bodenmikroorganismen, war bisher nicht bekannt. Um dies zu testen, ist ein Olfaktometer-System konstruiert worden, mit dem das Anlocken von Bodenbakterien über freigesetzte VOCs durch Wurzeln der Sand-Segge (*Carex arenaria*) analysiert werden konnten (Kapitel 7). Darüberhinaus ist untersucht worden, ob eine Pilzinfektion im Wurzelbereich eine Auswirkung auf die Bildung von VOCs und das Anlocken von bestimmten Bakterien hat. Der Versuch hat gezeigt, dass die Migration von entfernten Bakterien in Richtung der Wurzeln durch Wurzel-VOCs stimmuliert werden kann. Zudem hat eine im Versuch herbeigeführte Pilzinfektion eine Veränderung der Zusammensetzung von freigesetzten Wurzel-VOCs verursacht, was zum Anlocken von Bakterien mit pilz-inhibierenden Eigenschaften geführt hat. Versuche mit verschiedenen synthetisch hergestellten VOCs haben belegt, dass diese in der Tat über lange Distanzen im Boden diffundieren können, wobei die Diffussionsdistanz je nach Art der chemischen Zusammensetzung der volatilen Substanz variieren kann. Auf Basis der Ergebnissen dieser Teilstudie kann geschlussfolgert werden, dass pflanzliche VOCs eine bedeutsame Rolle in Pflanzen-Mikroben-Wechselwirkungen über lange Distanzen im Boden spielen können.

Insgesamt wurden mit dieser Doktorarbeit neue Erkenntnisse zur ökologischen Bedeutsamkeit von VOCs in Interaktionen zwischen Mikroben sowie Pflanzen und Mikroben im Boden aufgezeigt. Die Ergebnisse dieser Doktorabeit tragen somit zur Erweiterung des bisherigen Wissens über die Verbreitung und die ökologischen Funktionen von durch VOCs bedingten Wechselwirkungen zwischen Bodenorganismen bei.

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This PhD thesis is printed climate & resource neutral by planting three trees in Madagaskar via Naturefund e.V.

Curriculum Vitae

Kristin Bohm (née Schulz) was born on 23rd March 1988 in Dresden (Germany). During regularly stays at a camp side in her childhood, she got early inspired by nature. After finishing high school in 2006, she decided to study biology at the University of Bayreuth and got fascinated by the small things in nature. To get more insights into the ecology of microbes she conducted her Bachelor thesis at the Department of Ecological Microbiology in the group of Harold Drake. In 2009, Kristin went for an internship to England. In the group of Colin Murrel at Warwick University, she studied the ecology of alkane and alkene oxidizing soil bacteria. This was followed by a small internship in the group of Ralf Conrad at the Max-Planck-Institute of Terrestrial Microbiology. In 2010, Kristin continued her master studies at the University of Bayreuth and stayed affiliated with microbial ecology. In the group of Harold Drake and in collaboration with Siu M. Tsai and Carlos C. Cerri at CENA in Piracicaba (Brazil) she performed her Master thesis on the gut microbiology of greenhouse-gas emitting earthworms in Brazil. Missing to work in an international environment, Kristin decided to apply for a PhD position at the Netherlands Institute of Ecology. In 2013, she started her PhD in the group of Paolina Garbeva and came across the importance of chemical (volatile-mediated) communication in soil microbial ecology. After her PhD, Kristin would like to stay affiliated with research but with emphasis on applied-oriented fields.

Publications

Schulz-Bohm, K., Gerards, S., Hundscheid, M. P. J., Melenhorst, J., De Boer, W., & Garbeva, P. V. (2018). Calling from distance: Attraction of soil bacteria by plant root volatiles. *ISME Journal*, Online. doi:10.1038/s41396-017-0035-3

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Schulz-Bohm, K., Tyc, O., De Boer, W., Peereboom, N., Debets, F., Zaagman, N., Janssens, T.K.S., & Garbeva, P. (2017). Fungus-associated bacteriome in charge of their host behaviour. *Fungal Genetics and Biology*, 102, 38-48. doi:10.1016/j.fgb.2016.07.011

Schulz-Bohm, K., Geisen, S., Wubs, E. R. J., Song, C., De Boer, W., & Garbeva, P. V. (2017). The prey's scent – Volatile organic compound mediated interactions between soil bacteria and their protist predators. *ISME Journal*, 11, 817-820. doi:10.1038/ismej.2016.144

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Depkat-Jakob, P.S., Hunger, S., Schulz, K., Brown, G.G., Tsai, S.M., Drake, H.L. 2012. Emission of Methane by Eudrilus eugeniae and Other Earthworms from Brazil, *Applied and Environmental Microbiology* 78: 3014-3019

PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (4.5 ECTS)

- Competitive strategies of soil bacteria in fungal-rich and fungal-poor rhizosphere

Writing of project proposal (4.5 ECTS)

- Competitive strategies and competitive strength of bacteria in fungal-rich and fungal-poor rhizosphere environments

Post-graduate courses (1.9 ECTS)

- Microbial ecology; PE&RC (2015)
- Introduction to R for statistical analysis; PE&RC (2014)
- Programming in Matlab; BioSB (2017)

Invited review of (unpublished) journal manuscript (5 ECTS)

- FEMS Microbial Ecology: plant-microbe interactions (2016)
- FEMS Microbial Letters: microbial community composition in the rhizosphere of crop plants (2016)
- Frontiers in Microbiology: methodology to study VOCs-mediated interactions in soil (2016)
- Phytopathology: methodology of RT-PCR (2016)
- FEMS Microbial Letters: composition of fungal community in the rhizosphere of crop plants (2017)

Deficiency, refresh, brush-up courses (1.5 ECTS)

- Basic statistics; PE&RC (2014)

Competence strengthening / skills courses (3 ECTS)

- Competence assessment; WGS (2013)
- Information literacy including endnote introduction; WUR Library (2013)
- Scientific integrity; WGS (2013)
- Entrepreneurship in and outside science; WGS (2014)
- Voice matters: voice and presentation skills; WGS (2015)
- Brain training; WGS (2015)

PE&RC Annual meetings, seminars and the PE&RC weekend (3.7 ECTS)

- PE&RC Day: biomimicry (2013)
- PE&RC Day (2014)
- PE&RC Weekend (2014)
- One-day symposium: vegetation - soil interactions symposium (2014)
- Last stretch of the PhD programme (2016)
- NAEM conference; poster presentation (2016)
- NAEM Conference; poster presentation & organising/chairing of a session (2017)

Discussion groups / local seminars / other scientific meetings (9 ECTS)

- ME-Meeting with seminar (2013-2017)
- NIOO PhD literature discussion group (2013-2017)

International symposia, workshops and conferences (13.2 ECTS)

- Jacque Monod conference; poster presentation; Roscoff (2013)
- 4th International student conference on microbial communication; poster presentation; Jena (2014)
- 10th PGPR workshop; oral presentation; Liege (2015)
- Rhizosphere; poster presentation; Maastricht (2015)
- ISME; oral presentation and poster presentation; Montreal (2016)

Lecturing / Supervision of practicals / tutorials (3 ECTS)

- Ecological aspects of biological interactions (2016)

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