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Preface

Volatiles are ubiquitous. It is common knowledge that plants synthesise a diverse array of volatile compounds that mediate a variety of interactions between plants and their environment, ranging from attracting pollinators to protecting the plants from pathogens, parasites and herbivores. The role of volatile compounds in plant–microbe interactions was seldom reported prior to 2003. Since then, many studies showed that bacterial volatiles can promote plant growth and immunity, but also influence the growth of fungi. These reports triggered a strong interest in the scientific community and many researchers started to work on the topic of bacterial volatile emission and its relevance for agronomy, and more recently, also for the medical field, e.g. for disease diagnosis.

This book presents a comprehensive review about all major aspects of bacterial volatiles and their interactions with other organisms such as plants and fungi. The 14 chapters are written by leading experts of this young research field. In each chapter, basic concepts are presented and then recent findings are discussed. Chapters 1 and 2 are general introductions and overviews of bacterial volatiles as useful metabolites. The diversity of bacterial volatiles and detection technologies for the diagnosis of plant and human diseases are described in Chaps. 3 and 4. Chapter 5 deals with plant recognition of bacterial volatiles, which will be a pioneering area in this research area. Chapters 6–10 discuss plant health improvements and ecological aspects of bacterial volatiles under *in vitro* and field conditions. Chapter 11 describes volatiles emitted by the photosynthetically active cyanobacteria. Basic protocols on the collection and identification of bacterial volatiles are reviewed in Chap. 12. Finally, Chaps. 13 and 14 describe the application and formulation technologies provided for farmers.

The potential readers of this book include undergraduate students, graduate students or established researchers who want to know the state of the art of this

emerging research area, understand basic concepts, learn about detection, determination and application techniques of bacterial volatiles as well as researchers seeking innovative and sustainable solutions to agricultural or medical problems, respectively.

Daejeon, Republic of Korea Choong-Min Ryu

Contents

Chapter 1
The Domain of Bacteria and Their Volatile Metabolic Potential

Marie-Chantal Lemfack, Hubert Bahl, Birgit Piechulla, and Nancy Magnus

Abstract The ability to produce volatile compounds is widely distributed among bacteria. A comprehensive summary of volatiles reported to be emitted by different fungal and bacterial species can be found in the *mVOC database* (http://bioinformatics.charite.de/mvoc). This chapter aims to summarize different features of bacterial phyla, classes, families, and genera present in the *mVOC database* and review the different habitats where volatile producers have been isolated from. As a result, bacteria belonging to the phylum of Proteobacteria were the most studied mVOC producers. Moreover, soil, marine environments, and the human body turned out to be the main isolation sources of the microbes compiled in the *mVOC database*. Additionally, general biosynthetic routes from the primary as well as secondary metabolism are presented which ultimately can result in the production of microbial volatile metabolites.

Keywords *mVOC database* · Phylogeny · Habitat · Volatile biosynthetic routes · Primary metabolism · Secondary metabolism

1.1 Phylogeny and Diversity of Bacteria Comprised in the *mVOC Database*

Life on Earth can be categorized into three different domains: (1) Archaea, (2) Bacteria, and (3) Eukarya. In 1990, Woese et al. were the first to introduce this new taxonomic rank which was found to be superior to the rank of kingdom. According to this model, all life derived from the *last universal common ancestor* (LUCA) which therefore represents the origin of life. Whereas bacteria and Archaea are closer related to LUCA, Eukarya are thought to have evolved later in the history of the earth (Fig. 1.1).

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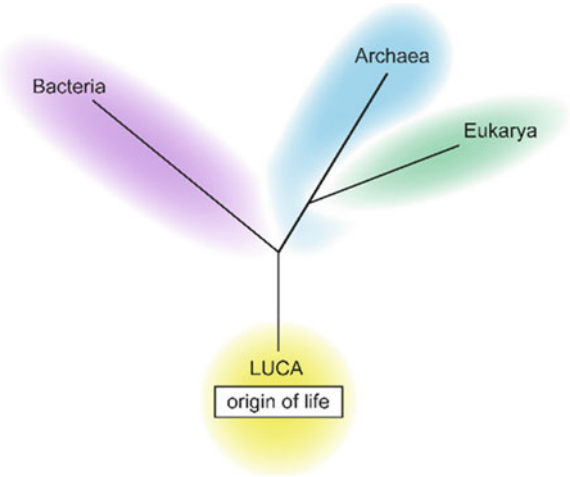

Fig. 1.1 The three domains of life. *LUCA* **l**ast **u**niversal **c**ommon **a**ncestor. Modified after Madigan et al. (2019)

Table 1.1 List of known bacterial phyla according to Parte (2018)

Phylum	Phylum
Acidobacteria	Elusimicrobia
Actinobacteria	Fibrobacteres
Aquificae	**Firmicutes**
Armatimonadetes	**Fusobacteria**
Bacteroidetes	Gemmatimonadetes
Balneolaeota	Kiritimatiellaeota
Caldiserica	Lentisphaerae
Calditrichaeota	Nitrospirae
Chlamydiae	Planktomycetes
Chlorobi	**Proteobacteria**
Chloroflexi	Rhodothermaeota
Chrysiogenetes	Spirochaetes
Coprothermobacterota	Synergistetes
Cyanobacteria	Tenericutes
Deferribacteres	Thermodesulfobacteria
Deinococcus–Thermus	Thermotogae
Dictyoglomi	Verrucomicrobia

Phyla found in *mVOC database* are indicated in bold

This chapter will only focus on the domain of bacteria. More specifically, main emphasis will be laid on phyla and genera present in the *mVOC database* (Lemfack et al. 2018). This database represents the first collection of all microbial volatile organic compounds investigated so far and comprises ca. 2000 compounds produced by roughly 1000 different microorganisms consisting of 600 bacterial and 400 fungal species (Lemfack et al. 2018). Bacterial VOC producers analyzed so far are distributed in 6 phyla (Table 1.1), 15 classes, 34 orders, 74 families, and 125 genera (Fig. 1.2). Since 10^{12} microbial species are expected to exist on Earth (Larsen et al.

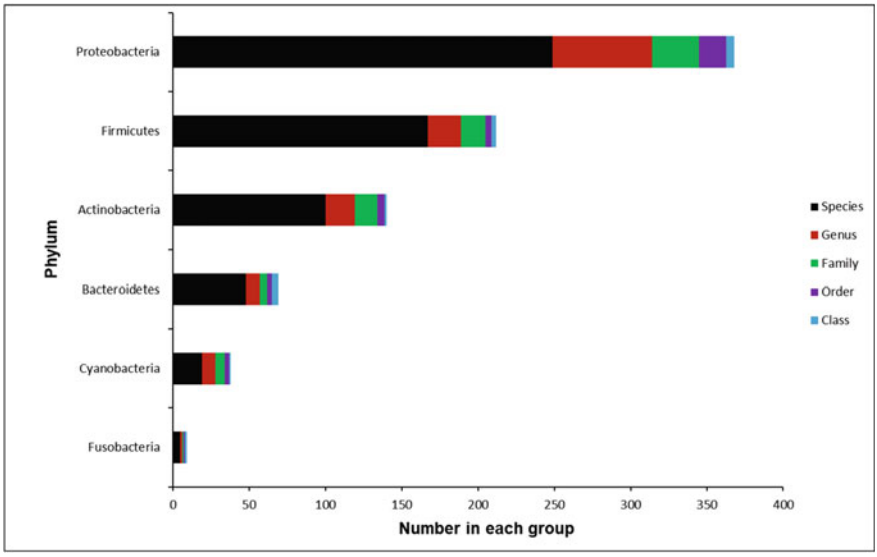

Fig. 1.2 Abundance of bacteria in *mVOC database*. The data represent the number of bacterial species, genera, families, orders, classes, and phyla compiled in *mVOC database* so far

2017; Pedrós-Alió and Manrubia 2016), the wealth of mVOCs still to be explored becomes obvious.

We will first summarize the main characteristics of bacterial phyla, classes, and genera present in the *mVOC database*, describe their distribution in nature and, furthermore, give a brief overview about the bacterial metabolism focusing on volatile end products.

The research field of bacterial phylogeny undergoes constant reconstructions which is why numbers mentioned in this chapter have to be treated with caution. According to the *List of prokaryotic names with standing in nomenclature* (LPSN, Parte 2018), the kingdom of bacteria currently comprises 34 different phyla which are listed in Table 1.1.

1.1.1 Proteobacteria

The Proteobacteria represent the largest and phenotypically most diverse division among prokaryotes (Gupta 2000). To date, more than 40 % of all validly published prokaryotic genera belong to this phylum (Kersters et al. 2006). They comprise the majority of Gram-negative bacteria and formerly have been referred to as "purple bacteria and relatives" although only a small fraction possesses purple coloration. This is why the phylum was renamed after the Greek god *Proteus* who could take on different shapes which reflects the diversity inside this phylum (Murray et al. 1990;

Stackebrandt et al. 1988). They consist of more than 200 different genera subdivided into eight classes based on 16S and 23S rRNA sequences: (1) Alphaproteobacteria, (2) Betaproteobacteria, (3) Gammaproteobacteria, (4) Deltaproteobacteria, (5) Epsilonproteobacteria, (6) Acidithiobacillia, (7) Hydrogenophilalia, and (8) Oligoflexia (Boden et al. 2017; Garrity et al. 2005; Nakai et al. 2014; Williams and Kelly 2013). Nevertheless, this classification does not reflect specific morphological or physiological traits that members of the same class have in common (Rizzatti et al. 2017). In contrast, Proteobacteria are very diverse concerning their appearance. They can form rods or cocci and curved, spiral, ring-shaped but also filamentous and sheathed cells. Most of them are motile and possess polar or peritrichous flagella. A special exception is found in the myxobacteria (Deltaproteobacteria) which possess a gliding motility (Kersters et al. 2006). The proteobacterial energy metabolism is also highly diverse including phototrophic genera (e.g., *Chromatium, Rhodospirillum*) as well as chemoorganotrophs (e.g., *Escherichia coli*) and chemolithotrophs (e.g., *Nitrobacter*) (Kersters et al. 2006).

Proteobacteria can be found ubiquitously in nature and are of high biological relevance since they include a vast number of human, animal, and plant pathogens (e.g., *Neisseria, Pseudomonas, Shigella*, *Salmonella, Yersinia, Escherichia, Helicobacter* spp.) (Bergey and Holt 2000; Collier et al. 1998; Dworkin et al. 2006; de Vos et al. 2009). Moreover, they represent one of the most abundant phyla in the gut microbiota (Rizzatti et al. 2017). Their cells mostly appear as free-living organisms with some exceptions. The most famous example is the genus *Rhizobium* which lives in symbiosis with leguminous plants. This Alphaproteobacterium is capable to reduce atmospheric nitrogen to ammonia which the host plants can readily assimilate. In turn, the plant provides the bacteria with photosynthetic products as a nutrient source. But Proteobacteria also appear as intracellular endosymbionts of protozoa and invertebrates (e.g., mussels, insects, and nematodes) (Kersters et al. 2006). Furthermore, it is anticipated that the origin of mitochondria can be traced back to the Alphaproteobacteria (Kersters et al. 2006).

When analyzing Proteobacteria present in *mVOC database* in detail, it appears that ca. 250 bacterial strains are described as VOC producers and are distributed in all five classes (Fig. 1.2). They are largely dominated by Gammaproteobacteria (150 strains) compared to Beta-(49), Alpha-(29), Delta-(29), and Epsilonproteobacteria (5) (Fig. 1.3a), while there are no data for Acidithiobacillia, Hydrogenophilalia, and Oligoflexia. Nevertheless, these last classes are composed of only few bacterial genera with validly published names. The most investigated genus concerning its VOC emission is *Pseudomonas,* accounting for one-third of all Gammaproteobacteria listed in the *mVOC database* (Fig. 1.3d) which is not surprising due to its high clinical relevance (summarized in Lyczak et al. 2000) raising the need for new, minimally invasive diagnostic tools. Other representatives of the Gammaproteobacteria which have been studied regarding their volatile emission are the genera *Serratia* and *Escherichia*, belonging to the family of *Enterobacteriaceae*. Both genera have been described to be good VOC producers (Bunge et al. 2008; Kai et al. 2010; Kai and Piechulla 2010; Umber et al. 2013; Weise et al. 2014; Yu et al. 2000; Zhu and Hill 2013) and important plant growth

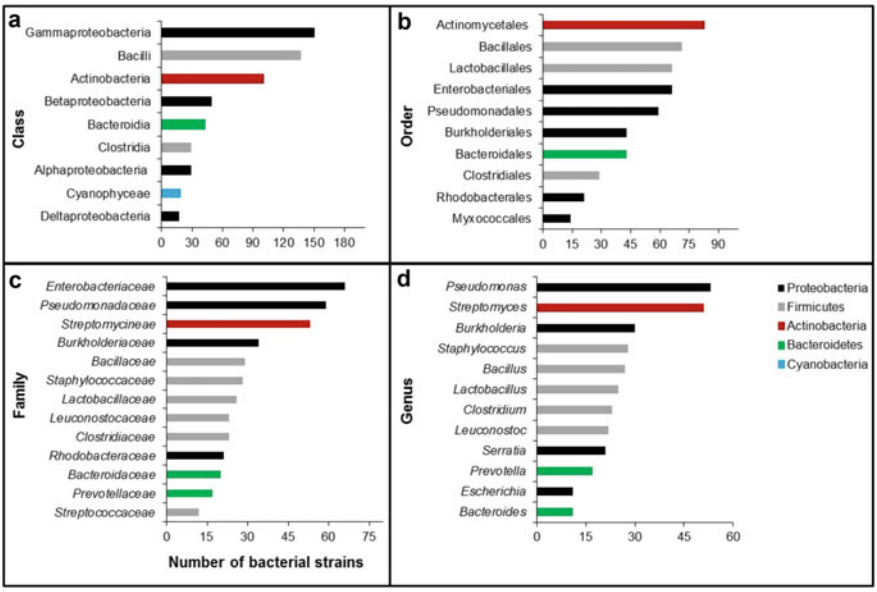

Fig. 1.3 Distribution of bacterial strains present in *mVOC database*. The data represent the number of bacterial strains (at least 10) present in each class (**a**), order (**b**), family (**c**), and genus (**d**) compiled in *mVOC database* so far. All classes, orders, families, and genera with less than 10 bacterial strains are not shown

promoting microorganisms (Devi et al. 2016; Nautiyal et al. 2010; Zaheer et al. 2016) which is why it is even more surprising to see that they are considerably less represented in the *mVOC database*. This also applies to the important plant symbiont *Rhizobium* having only one entry in the database.

1.1.2 Firmicutes

The Firmicutes represent the second largest phylum in the *mVOC database*. Ca. 170 bacterial strains have been investigated concerning their volatile emission, so far.

Firmicutes mostly consist of low G/C-containing (<50 %), Gram-positive bacteria with rigid or semi-rigid cell walls containing peptidoglycan (Gibbons and Murray 1978). Nevertheless, this phylum also contains single Gram-negative members, e.g., *Veillonellaceae* and *Syntrophomonadaceae*. But just like the Proteobacteria, Firmicutes are phenotypically very diverse reflected by high fluctuations in the composition of this phylum (Seong et al. 2018). Diversity is displayed in terms of (1) cell appearance (spherical, straight, curved, helical rods or filaments, flagellated or not, spore-forming as well as non-sporulating forms), (2) metabolism (chemoorganotrophic or anoxygenic photoheterotrophs), and (3) way of life

(aerobic, facultative or strict anaerobes, meso-, thermo- and halophiles, growth at neutral pH but also some alkali- and acidophiles) (de Vos et al. 2009). Bacteria belonging to the Firmicutes mainly divide by binary fission and are highly abundant in soil and aquatic environments (Baik et al. 2008; Schleifer 2009). They play a central role in the decomposition of organic matter which is why they are also often part of the normal intestinal flora of mammals (Lee et al. 2009). Nevertheless, they can also lead to the development of severe diseases in humans and animals as well as in plants (Nguyen and Götz 2016).

This large phylum is separated into three different classes: (1) "Bacilli," (2) "Clostridia," and (3) Erysipelotrichia (de Vos et al. 2009). Originally, the class of Mollicutes was also included, but due to low support using alternative markers and unique phenotypic properties, it was removed from the Firmicutes later on to form a phylum on its own (Ludwig and Schleifer 2005). According to the *mVOC database*, the class of "Bacilli" is the most investigated among Firmicutes (137 bacterial strains). "Bacilli" contain only Gram-positive members and can be divided into two orders, Bacillales and Lactobacillales. The most important genera of the Bacillales are *Staphylococcus* and *Bacillus* which are also the most investigated Firmicutes concerning their VOC emission with 28 and 27 bacterial strains, respectively.

Bacillus spp. are aerobic or facultative anaerobic, motile, endospore-forming bacteria. They can form rod-shaped, straight, or slightly curved cells and are highly heterogenous concerning their physiology, ecology, and genetics (Logan and de Vos 2009; Slepecky and Hemphill 2006). *Bacillus* is a very well investigated organism which has been known to mankind for long time, since its life cycle was first described already in 1872 by Cohn.

According to Lory (2014), the genus *Staphylococcus* currently contains 54 species. *Staphylococcus* spp. are widely distributed and are frequently found in association with human and animal hosts. Their appearance resembles grape-like clusters resulting from perpendicular division planes during cell division of single cocci (Lory 2014). They are facultative anaerobes which prefer to use respiratory pathways for energy generation rather than fermentation (Lory 2014). A prominent representative of the *Staphylococcus* genus is *Staphylococcus aureus* which can cause major infections in humans reaching from abscesses and food poisoning to endocarditis, toxic shock syndrome, and pneumonia (Gordon and Lowy 2008; Kravitz et al. 2005). Especially the methicillin-resistant *S. aureus* (MRSA) poses big challenges to medical research, due to increased fitness of the pathogen, improved evasion mechanisms of the hosts' immune system, and unique toxin production (Gordon and Lowy 2008).

From the order Lactobacillales, 25 *Lactobacillus* and 22 *Leuconostoc* species have been investigated for VOC emission (Fig. 1.3d). Both genera are Gram-positive, catalase negative, belong to the lactic acid bacteria, and thus are chemoorganotrophs (Schleifer and Ludwig 1995). They grow anaerobically but can also thrive well under microaerophilic conditions and prefer slightly acidic growth conditions (pH 5.5–6.5) (Schleifer and Ludwig 1995). Whereas the genus *Lactobacillus* contains at least 13 motile species (Neville et al. 2012; Puertas et al.

2014), *Leuconostocaceae* were characterized as non-motile and are often found in nutrient-rich environments, e.g., green vegetation, roots, and food (Nieminen et al. 2014).

The second major class of Firmicutes found in the *mVOC database* is the "Clostridia." In total, only 29 bacterial strains of this class have been investigated regarding their volatile emission so far. The data further indicate that the *Clostridiaceae* and more specifically the genus *Clostridium* account for 23 of the aforementioned 29 "Clostridia"-entries in *mVOC*. Members of the *Clostridiaceae* contain more than 30 genera that can be found ubiquitously in nature which is not surprising since they can form endospores protecting them from different kinds of stresses. They are Gram-positive, rod-shaped cells that live anaerobically and contain meso-diaminopimelic acid in their peptidoglycan which is used as a diagnostic marker (Stackebrandt 2014).

As already mentioned above, several Firmicutes are known pathogens and it appears in general that only a very low number of pathogenic bacteria were analyzed for mVOC production. Genera like *Streptococcus,* responsible for oral malodor, are almost not represented (Fig. 1.3d).

1.1.3 Actinobacteria

The Actinobacteria are one of the largest taxonomic units among the major lineages in the domain of bacteria composed of Gram-positive bacteria with high G/C content and typical filamentous morphology (Barka et al. 2015; Dhakal et al. 2017; Ludwig et al. 2012a). They contain mostly free-living organisms which can be found in terrestrial as well as in aquatic and marine ecosystems (Macagnan et al. 2006). In soil, *Streptomyces* spp. are predominantly found, whereas *Salinispora* spp. are a prominent example for Actinobacteria living in aquatic environments. Moreover, bacteria of this phylum also live in close symbiosis to plants, like *Frankia* spp., or can also have pathogenic effects on plants as well as animals (e.g., *Corynebacterium*, *Mycobacterium,* and *Nocardia* spp.) (Barka et al. 2015; Macagnan et al. 2006). Actinobacteria, more specifically members of the genus *Bifidobacterium*, also affect us humans, since they are among the first bacteria to colonize our intestinal tract and are believed to exert positive health effects although the molecular mechanisms are still not understood (Cronin et al. 2011; O'Callaghan and van Sinderen 2016). Most Actinobacteria live aerobically under mesophilic conditions (optimal temperature between 25 and 30 °C) at a pH ranging from 6 to 9 with an optimum at neutral pH values (Barka et al. 2015).

Phylogenetically, this phylum is divided into six classes: (1) Actinobacteria, (2) Acidimicrobiia, (3) Coriobacteriia, (4) Nitriliruptoria, (5) Rubrobacteria, and (6) Thermoleophilia. The class of Actinobacteria comprises 16 orders including Actinopolysporales, Actinomycetales, Bifidobacteriales, Catenulisporales, Corynebacteriales, Frankiales, Glycomycetales, Jiangellales, Kineosporiales, Micrococcales, Micromonosporales, Propionibacteriales, Pseudonocardiales,

Streptomycetales, Streptosporangiales, and Incertae sedis (Ludwig et al. 2012b). Due to its large size, it is not surprising that members of this phylum are highly diverse in terms of morphology, physiology, and metabolic capabilities. Concerning morphology, most Actinobacteria form substrate mycelia in liquid and solid cultures which during growth on solid medium can transform into aerial mycelia to produce asexual exospores (Flärdh and Buttner 2009; van Dissel et al. 2014). Nevertheless, cell shapes can range from coccoid (*Micrococcus*), rod-coccoid (*Arthrobacter* spp.), and fragmented (*Nocardia*) to permanent and highly differentiated, branched mycelia (*Streptomyces, Frankia*) (Atlas 1997). Often, Actinobacteria are discriminated according to their mycelium color since most strains are capable to produce melanoid pigments which can improve survival and competitiveness of the producers. Additionally, the appearance and structure of exospores can be used to differentiate actinobacterial species (Barka et al. 2015). The most common soil-dwelling genera found are the saprophytic *Streptomyces*, already accounting for 70%, *Nocardia* and *Micromonospora* (Yokota 1997) which play important roles in the decomposition of organic matter, e.g., cellulose and chitin. As a result, they are crucial for the carbon fluxes in nature (Tarkka and Hampp 2008). Moreover, *Streptomyces* species are used extensively for the discovery of new bioactive secondary metabolites and produce 70–80% of all substances applied in pharmacy or agrochemistry, e.g., antibiotics like actinomycin or streptomycin (Bérdy 2005; Hopwood 2007; Manteca et al. 2008). Secondary metabolite production is most efficient during morphological differentiation of the cells, i.e., during transition from vegetative to aerial growth (Bibb 2005; Granozzi et al. 1990). So far, more than 500 species have been described in the genus *Streptomyces* (Tarkka and Hampp 2008) of which only 51 were also investigated regarding their volatile emission. Summarizing everything, although Actinobacteria are ubiquitous and a rich source of natural products, they are less investigated concerning volatile emission compared to other phyla or classes with *Streptomyces* being the most investigated genus, while other genera like *Corynebacterium* or *Actinomyces* are significantly less represented in the data compiled so far (Figs. 1.2 and 1.3).

1.1.4 Bacteroidetes

The phylum of Bacteroidetes is composed of Gram-negative, rod-shaped, chemoorganotrophic bacteria which form no spores and are mostly non-motile or possess a gliding motility (Hahnke et al. 2016; McBride and Zhu 2013; Paster et al. 1994; Woese 1987). It includes ca. 7000 species (Thomas et al. 2011) which are, according to the Bergey's Manual of Systematic Bacteriology, distributed into four different classes: (1) Bacteroidia, (2) Cytophagia, (3) Flavobacteriia, and (4) Sphingobacteriia (Krieg et al. 2011a). Bacteroidetes are widespread in nature and can be found in soil, decaying plant material, freshwater, marine environments, algae, and dairy products (Bernardet and Nakagawa 2006; Reichenbach 2006). Nevertheless, different classes of Bacteroidetes are also distributed differentially.

Accordingly, strains of the class Cytophagia were found only in marine habitats or soil, whereas Flavobacteriia colonize more diverse ecosystems, like soil, sediment, freshwater, brackish water, or seawater including some pathogenic species for humans, mammals, and fish (Bernardet 2015; Krieg et al. 2011b). Just like the Actinobacteria, environmental Bacteroidetes are thought to play a role in the decomposition of complex organic matter, e.g., of polysaccharides and proteins (Bowman 2006; Church 2008; Fernández-Gómez et al. 2013; Kirchman 2002). Even more importantly, Bacteroidetes are found in the gastrointestinal (GI) tract of animals as well as humans. Here, they can account for up to 50% of the microbial gut flora which makes them the dominating phylum together with the Firmicutes (Eckburg et al. 2005; Ley et al. 2008; Smith et al. 2006; Thomas et al. 2011). Moreover, recent studies indicated that the ratio between Firmicutes and Bacteroidetes in the gut microbiota raised with increasing *body mass index* (BMI) suggesting a role of Bacteroidetes in obesity (Barlow et al. 2015; Koliada et al. 2017; Sweeney and Morton 2013).

In terms of volatile emission, Bacteroidetes are rather unexplored so far. Only 48 entries (<10%) can be found in the *mVOC database* (Fig. 1.2), most of which belong to the class of Bacteroidia (43 bacterial strains) with the main genera being *Bacteroides* (20 strains) and *Prevotella* (17 strains) (Fig. 1.3a, d). Both, *Bacteroides* spp. and *Prevotella* spp. contain solely anaerobic bacteria, in contrast to the other three Bacteroidetes classes which comprise only aerobic or facultative anaerobic species. Species of the genus *Bacteroides* are generally considered as friendly commensals, although the term mutualism is much more fitting since both partners are benefitting from each other (Bäckhed et al. 2005). Moreover, this genus alone accounts for ca. 25% of the total human gut microbiota (Wexler 2007). *Bacteroides* spp. become a part of the human microbiome already very early in life and can be passed from mother to child during vaginal birth making a crucial role for the human body apparent (Reid 2004). Specifically, it has been shown that *Bacteroides* spp. produce volatile fatty acids which can be reabsorbed from the host through the large intestine and, in turn, be used as an energy source (Hooper et al. 2002). Nevertheless, when *Bacteroides* escape their native niche, for example, through rupture of the GI tract or surgical interventions, they can cause severe pathologies outside the gut, like formation of abscesses or bacteremia (summarized in Wexler 2007). The genus *Prevotella* basically fulfils equal functions in the human host. The only difference is that *Prevotella* spp. are associated with a plant-rich diet as well as with chronic inflammatory conditions which is why gut microbial communities contain either *Bacteroides* or *Prevotella*, but not both at the same time (Brook 1998, 2004; Ley 2016; Nagy 2010).

1.1.5 Cyanobacteria

Cyanobacteria are thought to have emerged ca. 3 Ga which was when the transition to oxygenic conditions through the evolution of photosynthesis took place

(Schirrmeister et al. 2011). They belong to the most diverse and widely distributed Prokaryotes and can be found in freshwater, marine, terrestrial, planktonic, and benthic habitats as well as in extreme environments, i.e., frozen lakes, hot springs, or salt works (Walter et al. 2017; Whitton 1992). Cyanobacteria are usually Gram-negative but appear much larger than most bacteria ranging from 1 μm for unicellular forms to more than 30 μm for filamentous forms (Singh and Montgomery 2011). In general, species of this phylum are oxygenic phototrophs which makes them independent from specific carbon sources like other bacteria. Nevertheless, also species growing heterotrophically on organic compounds have been reported (Halm et al. 2012; Walter et al. 2017). Furthermore, some secondary metabolites produced by cyanobacteria are potentially toxic and can lead to harmful effects on health during algal blooms (Percival and Williams 2014). Morphologically Cyanobacteria are very diverse. They exist either as unicellular or filamentous forms whereby also the unicells can be bound together by mucilaginous secretions resulting in complex formations (Broady and Merican 2012; Singh and Montgomery 2011). These complexes can also become very large which form mats, crusts, or gelatinous masses on rocks, sediments, soil, and vegetation getting visible with the naked eye (Broady and Merican 2012). Moreover, single cells can differentiate into specialized compartments like heterocysts or akinetes. Heterocysts are rounder, thick-walled cells that are capable to fix atmospheric nitrogen and produce ammonia which can further be used for amino acid biosynthesis making it an available N-source for the surrounding vegetative cells and thus promote growth during nitrogen starvation (Kumar et al. 2010; Meeks and Elhai 2002). Akinetes represent thick-walled resting spores which secure survival of the population under worst conditions (Walter et al. 2017). Moreover, some cyanobacterial species can produce gas vacuoles which allow them to float in aquatic environments or exhibit a gliding motility (Hoiczyk 2000; Lyra et al. 2005; Percival and Williams 2014).

In total, the phylum Cyanobacteria includes ca. 150 genera containing about 2000 species (Percival and Williams 2014). They can be divided into five different orders: (1) Chroococcales, (2) Pleurocapsales, (3) Oscillatoriales, (4) Nostocales, and (5) Stigonematales (Waterbury 2006). In *mVOC* only a total of 17 validated cyanobacterial strains have been investigated for their volatile emission profiles. Most of them belong to the orders Nostocales (8 strains) and Oscillatoriales (8 strains) and one representative of the Chroococcales. Nostocales include filamentous cyanobacteria that are capable to differentiate into the aforementioned heterocysts and akinetes which makes them truly multicellular organisms since differentiation is irreversible and functional specialization takes place (Komárek and Johansen 2015; Waterbury 2006). They divide by binary fission in one plane at right angles to the long axis of the trichomes (Waterbury 2006). Species belonging to the order of Nostocales listed in *mVOC* are of the genera *Anabaena* (1), *Calothrix* (3), *Rivularia* (2), and *Tolypothrix* (2). Oscillatoriales also contain filamentous cyanobacteria which are, in contrast to Nostocales, not differentiated and divide by binary fission in a single plane. Genera found in *mVOC* are *Lyngbya* (1), *Phormidium* (1), *Plectonema* (3), and *Oscillatoria* (3). As already mentioned, there is only one representative of the order Chroococcales to be found in *mVOC*

database, *Spirulina platensis*. Generally, Chroococcales entail only unicellular cyanobacteria that reproduce by binary fission or budding occurring in one to three planes at right angles to one another or in irregular planes. Cell shapes range from single cocci and rods to cell aggregates held together by sheath material, amorphous slime, or capsular material (Waterbury 2006).

1.1.6 Fusobacteria

The Fusobacteria are a rather unexplored bacterial phylum. They consist of Gram-negative, anaerobic, non-sporulating, non-motile bacilli with tapered rod-shaped cells (Brennan and Garrett 2019). Naturally, Fusobacteria species can be found as free-living organisms in marine environments (*Ilyobacter* spp.) but also in the human oral cavity (*Fusobacterium* spp.), intestinal and urogenital tracts (*Leptotrichia* and *Sneathia* spp.) and in the intestines of fishes and whales (*Cetobacterium* spp.) (Brennan and Garrett 2019). Interestingly, single Fusobacteria can harbor unique metabolic capabilities, e.g., *Psychrilyobacter atlanticus* which was shown to break down nitramine explosives (Zhao et al. 2009). Members of this phylum are differentiated in two families: (1) *Leptotrichiaceae* and (2) *Fusobacteriaceae*. In *mVOC* only five representatives of the latter are found including three strains of the species *Fusobacterium nucleatum* besides one *F. necrophorum* and *F. simiae* species each.

Fusobacterium spp. are generally found in the mouth and other mucosal sites inside humans or animals. Their presence in healthy tissues suggest that they are natural constituents of the microbiota (Brennan and Garrett 2019). Especially the genus *F. nucleatum* caught scientific interest. It is most abundant in the oral cavity and was shown to play a central role in biofilms that contribute to periodontal health and disease. It mediates biofilm formation by serving as a bridge organism between primary (e.g., *Streptococcus* spp.) and secondary colonizers (e.g., *Porphyromonas gingivalis*) (Brennan and Garrett 2019; Kolenbrander et al. 2010). Nevertheless, *F. nucleatum* is also considered as an opportunistic pathogen since it was found to play a role during periodontitis by shaping host responses and increasing its infectivity by other pathogens (Binder Gallimidi et al. 2015). Furthermore, this species was also implicated to be involved in other diseases such as appendicitis (Swidsinski et al. 2011), brain abscesses (Han et al. 2003), osteomyelitis (Gregory et al. 2015), pericarditis (Truant et al. 1983), and chorioamnionitis (Altshuler and Hyde 1988) although the definite role of *F. nucleatum* in these cases remains unclear. Still, this species is known to promote inflammatory responses by binding/invading diverse cell types in the human body strengthening the notion of *F. nucleatum* as an opportunistic pathogen (Han et al. 2000; Ikegami et al. 2009; Strauss et al. 2011).

1.2 Natural Habitat of Bacteria in *mVOC Database*

Among the 600 bacterial strains registered in *mVOC database* so far, the isolation sites of ca. 500 of them were compiled, while there were no data existing in the literature for the 100 other strains. It turned out that most of the bacteria present in the database were isolated from the rhizosphere (17%), soil (15%), aquatic environment (15%), clinical samples (15%), humans (13.5%), food products (10%), animals (5%), plants (5%), plant waste (3%), and fungi (1.5%) (Fig. 1.4). Although the volatile profiles of microbes are independent of their isolation site, the number of bacteria isolated from animals and investigated for VOC emission remains very low. Likewise, only the volatile profiles of plant-associated bacteria like *Pseudomonas*, *Bacillus,* and *Burkholderia* are commonly studied (Piechulla et al. 2017). Most of these microorganisms are isolated from the rhizosphere/soil, while the upper parts of the plants as well as animal-associated bacteria (e.g., insects) still represent an important unexplored potential.

Altogether, these analyses of the *mVOC database* reveal that among the huge number of bacterial species existing on Earth (10^{12}; Larsen et al. 2017; Pedrós-Alió and Manrubia 2016) only a very small proportion, i.e., 0.00000006%, can presently be found in the database. Furthermore, it became obvious that from 26 known bacterial phyla (most of them listed in Fig. 1.1) only six have been investigated concerning their volatile emission, so far. Therefore, the plethora of bacteria which remain to be investigated represents a huge potential in the discovery of further information regarding their ecological role(s).

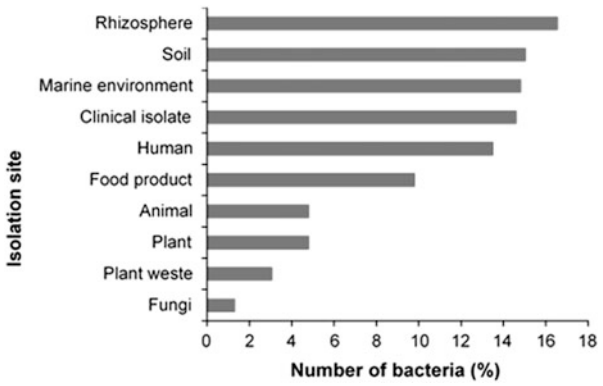

Fig. 1.4 Isolation site of bacteria described in *mVOC database*. All isolation sites with less than 1% of bacteria are not represented

1.3 Bacterial Volatiles Derived from the Primary Metabolism

The emission of volatiles by bacteria is known for decades. These volatiles are mainly generated during the catabolic activity of these microorganisms. In general, these substances are considered either as primary or as secondary metabolites, depending on whether they are produced during the exponential growth phase or during the transition to or in the stationary growth phase, respectively. The vast majority of volatiles is certainly formed during secondary metabolism. Despite their limited number, also primary metabolism volatiles such as CO_2 should not be neglected, e.g., when the interaction between organisms is determined (Piechulla 2017). In the following section, the formation of volatiles by bacteria during primary metabolism is described.

The diversity of bacteria is primarily not marked by a large variability in morphology but by a vast number of different metabolic, especially catabolic, pathways. Bacteria can make a living by generation of energy (ATP) for biosynthetic activity using either light (phototrophy) or the oxidation of chemical compounds (chemotrophy) as energy source. Besides organic material (chemoorganotrophy), a number of inorganic molecules can serve as electron donors for energy generation (chemolithotrophy). The carbon source of bacteria is either organic material or CO_2. In Table 1.2, the different types of chemotrophic metabolism and the main volatile products are depicted.

In general, during primary metabolism the ultimate ambition of the bacteria is to generate as much energy (ATP) for growth as possible. In addition, the amount of energy gained during chemotrophic metabolism (oxidation of the substrate) depends on the electron acceptor available or utilizable by a specific bacterium. Some bacteria are very flexible with this respect. Enteric bacteria such as *E. coli* are a good example for this. They can oxidize glucose, e.g., completely to the inorganic volatile CO_2 in the presence of oxygen. If no oxygen but nitrate is on hand, it carries out anaerobic respiration and reduces nitrate to NH_3, which also is released into the atmosphere. If no external electron acceptor is present, *E. coli* switches to fermentation and transfers the electrons to internal acceptors, which finally leads to the excretion of acetate, ethanol, and formate which are altogether organic volatiles (Fig. 1.5). Thus, with respect to the production of volatiles by bacteria the incomplete oxidation of an organic substrate is of special interest, since a greater variety of volatiles might be generated compared to the main volatile CO_2 as end product of complete oxidation. In addition to the main alternative electron acceptors nitrate and sulfate listed in Table 1.2, several other compounds can serve as electron sink during growth of certain bacteria, e.g., dimethyl sulfoxide (DMSO) is reduced to dimethyl sulfide and trimethyl amine-*N*-oxide (TMAO) to trimethyl amine (Fuchs et al. 2007; Madigan et al. 2017). A special metabolic type is the incomplete oxidation of the substrate despite the fact that oxygen is present. Here, a complete oxidation is not possible, since either, an enzyme of the tricarboxylic acid cycle is missing (acidic acid bacteria) or is repressed (bacilli) (Gottschalk 1986). Examples of an incomplete

Table 1.2 Major volatiles produced by bacteria during primary metabolism

Metabolic type	Electron donor	Electron acceptor	Inorganic volatiles	Organic volatiles	Example
Chemoorganotrophy					
Aerobic respiration	Carbohydrate	O_2	CO_2	–	*Pseudomonas aeruginosa*
	Protein, amino acids[a]	O_2	CO_2, NH_3, H_2S	Methanethiol dimethyl disulfide	*Micrococcus luteus*
Incomplete oxidation	Carbohydrate	O_2	CO_2	Acetoin, 2,3-butandiol, acetic acid, pyruvate	*Bacillus subtilis*
Anaerobic respiration[b]					
Nitrate respiration	Carbohydrate	NO_3^-	CO_2, NO, N_2O, N_2, NH_3		*Pseudomonas stutzeri*
Sulfate respiration	Lactic acid	SO_4^{2-}	H_2S	Acetic acid	*Desulfovibrio vulgaris*
Sulfur respiration	Acetic acid	S^0	CO_2, H_2S		*Desulfobacter curvatus*
Fermentation	Carbohydrate	Internal metabolite	H_2, CO_2	Alcohols, ketones, fatty acids[c]	*Clostridium acetobutylicum*
Chemolithotrophy					
Anaerobic respiration[b] nitrate respiration	H_2S	NO_3^-	NO, N_2O, N_2, NH		*Thiobacillus denitrificans*
Sulfate/sulfur respiration	H_2	SO_4^{2-}, S^0	H_2S		*Desulfobacterium autotrophicum*
Carbonate respiration	H_2, CO	CO_2		Acetic acid, ethanol CH_4[d]	*Clostridium aceticum*

[a]The volatiles listed here are likely be produced also in other metabolic types as long as proteins or amino acids are present in the medium as carbon or nitrogen source
[b]The list of electron acceptors for anaerobic respiration shown in this table is not complete, see text
[c]For an overview on bacterial fermentation products see Fig. 1.5
[d]CH_4 is not produced by bacteria but by methanogenic archaea

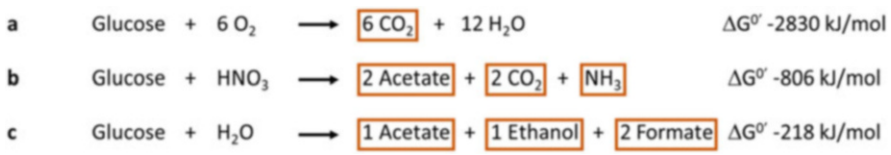

Fig. 1.5 Volatile production of facultative aerobic bacteria according to the electron acceptor used. (**a**) aerobic respiration (O_2 as electron acceptor); (**b**) anaerobic respiration (nitrate as electron acceptor); (**c**) fermentation (internal electron acceptor). Volatiles are highlighted by a red box

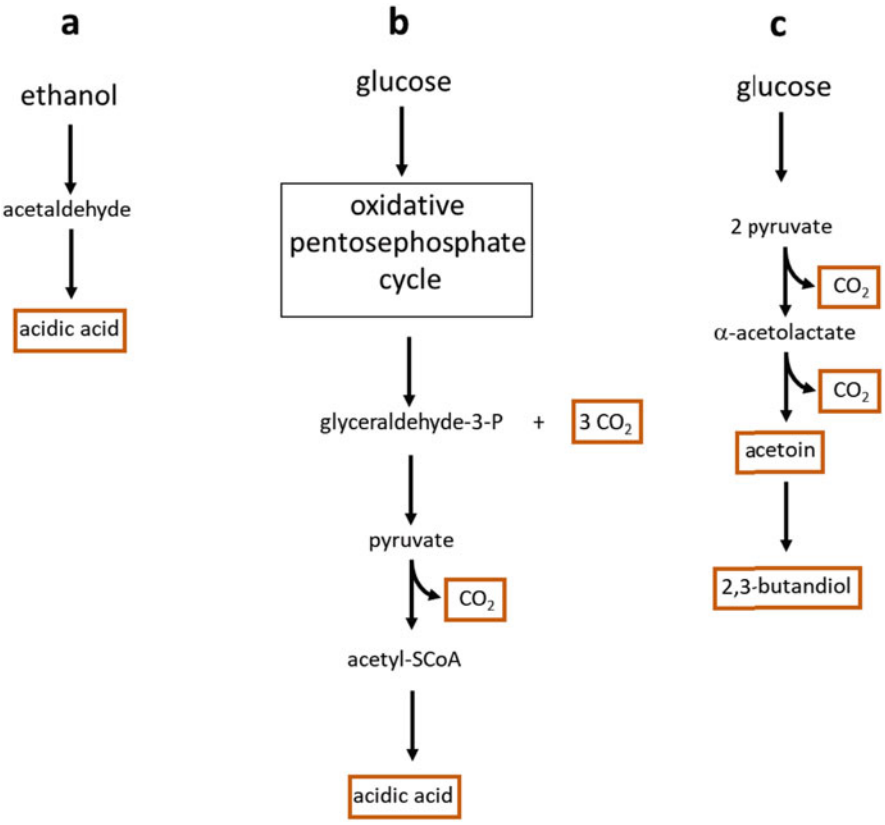

Fig. 1.6 Volatile production caused by incomplete oxidation of the substrate in the presence of oxygen. (**a**) incomplete oxidation of ethanol by acidic acid bacteria; (**b**) incomplete oxidation of glucose by acidic acid bacteria; (**c**) incomplete oxidation of glucose by *Bacillus subtilis*. Volatiles are highlighted by a red box

oxidation of glucose and ethanol and the formation of corresponding volatiles are shown in Fig. 1.6. Bacterial fermentation processes lead to the emission of a number of organic volatiles (alcohols, fatty acids, ketones) in addition to the gases CO_2 and H_2. Figure 1.7 demonstrates in more detail the diversity of volatiles produced from glucose by different fermentative bacteria. Especially clostridia carrying out butyric acid or an acetone–butanol fermentation can emit a rich bouquet of volatiles.

In summary, which volatiles (and other products) bacteria produce as primary metabolites depends on the substrate they grow on and on the growth conditions.

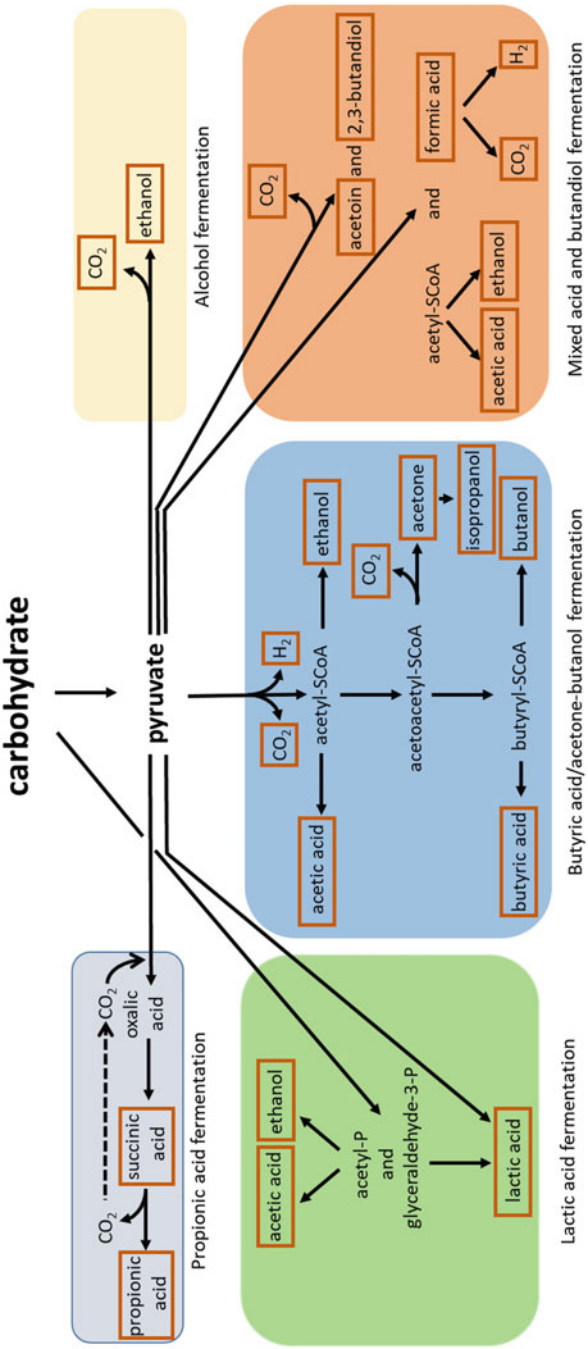

Fig. 1.7 Volatile production during fermentation. Volatiles are highlighted by a red box

1.4 Bacterial Volatiles Derived from the Secondary Metabolism

Many organisms produce metabolites which are not essential for the central processes of growth and development (= primary metabolism). These compounds are referred to as secondary or specialized metabolites, or natural products. They are often unique to individual species or groups of species and mediate interactions with other organisms (defense and attraction arsenal). Typical secondary metabolite classes found in plants are terpenes, cyanogenic glycosides glucosinolates, phenylpropanoids, alkaloids, fatty acid derivatives, S- and N-containing compounds. Chemical convergence of some biosynthetic pathways between plants and insects was documented recently (Beran et al. 2019), while chemical convergences between the microbial and plant and/or animal kingdoms are less well studied, despite the fact that microorganisms release a wealth of secondary metabolites.

One prominent ecological role of specialized compounds released by bacteria is to structure the microbial community and populations living in the same habitat. Many of these compounds are well-known antibiotics that are produced to inhibit the growth of different (microbial) species and are therefore often used in human health care. Most secondary metabolites have unusual structures and their biosyntheses are catalyzed by enzymes that are normally clustered on the chromosome and infrequently on plasmids (e.g., cyt P450 enzymes, glucosyltransferases). Despite the huge variety of chemical structures, the sequence of reactions by which they are made can be grouped into three polymerization reactions:

1. Condensation of acetate-malonate units (polyketide biosynthesis).
2. Condensation of amino acids to oligopeptides (non-ribosomal peptide biosynthesis).
3. Condensation of carbohydrate units (often amino sugars).

The polyketide biosynthetic pathway among prokaryotes is prominent in actinomycetes, but some polyketide compounds are also produced by myxobacteria, cyanobacteria, *Bacillus* sp., and pseudomonads. Polyketides produced by microorganisms show an extraordinary diversity (Helfrich et al. 2014; Jenke-Kodama and Dittmann 2009), despite its core biosynthesis based on repeated cycles of decarboxylative Claisen-like condensations of simple acyl-CoA building blocks which resemble fatty acid biosynthesis. A multienzyme complex (type II fatty acid biosynthesis) is present in bacteria and plants, while type I single multifunctional FAS are present in invertebrates. Polyketides are typically synthesized by type II PKS. Acetate and malonate or alternatively propionate and methylmalonate form chains in which the keto groups and methylene groups alternate. When methylmalonate is used instead of malonate, the chain becomes branched with methyl groups. The biosynthesis starts with acetyl CoA and malonyl CoA, both bound to the synthase as thioesters (Fig. 1.8a). Acetate (= initiator) binds to the condensing enzyme domain and malonate binds to acyl carrier protein (ACP). Acetate is condensed with the methylene carbon of malonate, while at the same

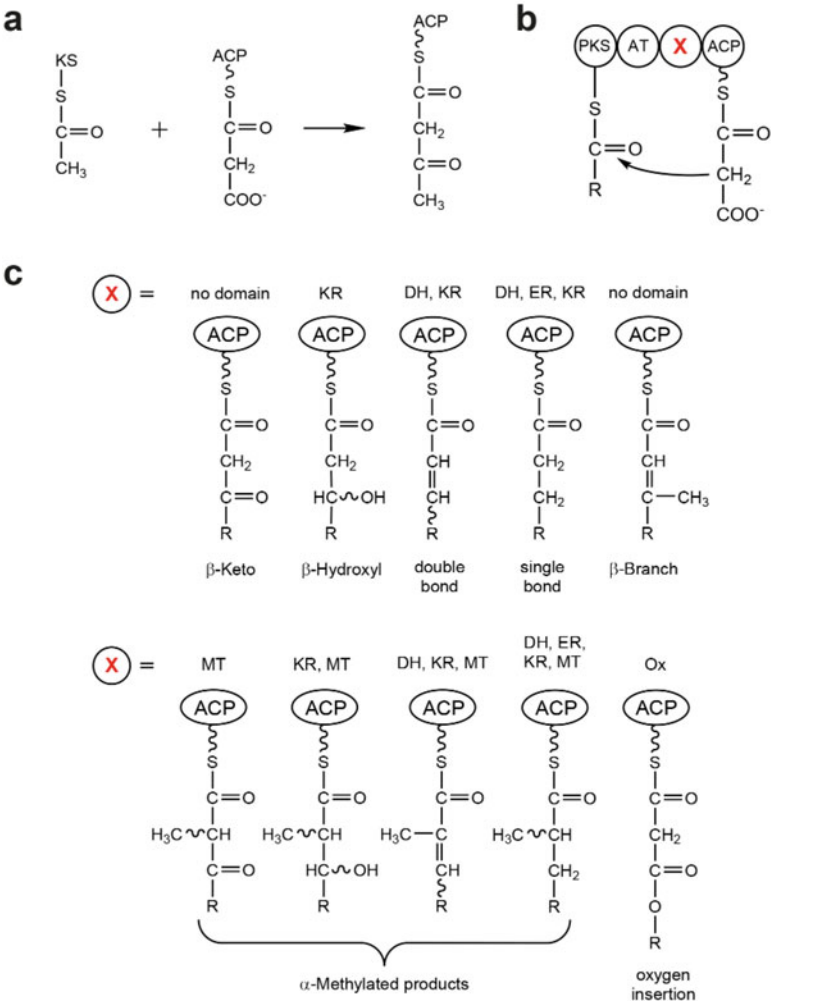

Fig. 1.8 Polyketide biosynthesis (PKS). (**a**) First condensation step of the classical fatty acid biosynthesis. (**b**) A single round of elongation in a type I *cis*-acyltransferase (AT) PKS module. Examples of modifications and derivatizations are introduced via various domains (red X). In *trans*-AT PKS, the AT and ER domains are usually missing. (**c**) Modification reactions, *DH* dehydratase, *ER* enoyl reductase, *KR* ketoreductase, *MT* methyltransferase, *Ox*: oxygenase. Modified based on Nguyen et al. (2008) and Meoded et al. (2018)

time the carboxyl group of malonate eliminates carbon dioxide resulting in an acetoacetate bound to ACP. In fatty acid biosynthesis three reactions reduce the keto group of acetoacetate to a methylene group. These steps are partially or totally omitted during secondary metabolite biosynthesis. Consequently, the chain can bear keto or hydroxyl groups or double bonds are formed adjacent to methylene groups.

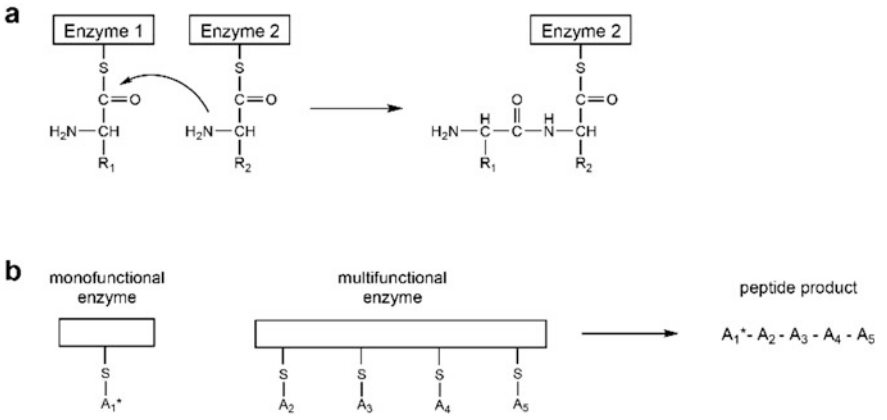

Fig. 1.9 Non-ribosomal peptide synthesis (NRPS). (**a**) Amino acids are linked via thioesters to mono- or multifunctional enzymes. (**b**) Amino group and carboxyl group of amino acid 2 and 1, respectively, form peptide bonds until the peptide is released by a thioesterase from the enzyme. The mono- as well as multifunctional enzymes may encompass domains which modify the amino acids, e.g., isomerization from L- to D isomer (*)

The extended chain is then transferred to the condensing enzyme and another malonate-ACP is used for chain elongation.

The complexity and diversification of polyketides is large and depends on (1) the selection of building blocks, (2) the facultative enzymatic modifications, and (3) additional activity of a variety of auxiliary enzymes during or after chain elongation (e.g., dehydratase, enoylreductase, ketoreductase, methyltransferase, oxygenase, Fig. 1.8c, Meoded et al. 2018; Nguyen et al. 2008). Depending on the nature of the enzymes involved as well as altered initiator and extender molecules, the chain can be converted by the aldol reaction into aromatic rings and either linear molecules or macrocyclic rings are formed, subsequently many different structures are produced, e.g., erythromycin, tetracycline, rifamycin, and monensin A. Polyketide synthases (PKS) also encompass different types regarding their enzyme architecture (non-modular, mono-modular, multi-modular) and mode of operations (iterative, non-iterative, *cis*- and *trans*-AT). They can also form hybrid enzymes containing components of different PKS classes and/or non-ribosomal peptide synthetases (NRPS) (Helfrich et al. 2014). Very recently, several putative Diels-Alderases (cycloaddition) have been characterized in PKS/NRPS pathways which act in tailoring events (summarized in Scott and Piel 2019).

The vast majority of peptide antibiotics are synthesized by the thiotemplate mechanism (*non-ribosomal protein biosynthesis*). This process starts with the activation of the amino acids as adenylates, followed by the condensation of the carboxyl group of the amino acid to thiol groups of the non-ribosomal peptide synthases (NRPSs) to form thioesters and stepwise polymerization. The polymerization initiates with the formation of a peptide bond between the carboxyl group of the first amino acid and the amino group of the second amino acid (Fig. 1.9a). These

condensation reactions are repeated until the chain is completed. A thioesterase releases the peptide. The NRPSs that catalyze this process can comprise up to four multifunctional enzymes, containing domains which catalyze the activation of an amino acid, its esterification to the thiol group of a pantetheine moiety and the formation of the peptide bond. The enzymes may also encompass domains that catalyze isomerization from L- to D-isomers or methylation of the nitrogen of a newly formed amide, resulting in, e.g., gramicidin biosynthesis (Fig. 1.9). This principle of this process resembles that of polyketide biosynthesis.

Antibiotics often contain sugar residues. The biosynthesis of oligosaccharides is identical with the polysaccharide biosynthesis of bacterial cell walls, they are formed by the assembly of monomers, activated as nucleoside diphosphates at the anomeric carbon. Unusual oligosaccharides, often present in antibiotics, either are first assembled and then modified, or sugars are first modified and then stepwise condensed to the precursor.

In contrast to the well-known and established biosynthesis pathways for high molecular weight compounds in microorganisms/bacteria, the biosyntheses of some small molecular compounds of microorganisms, such as terpenes and pyrazines, are less well studied.

Presently, ca. 2000 VOCs released from microorganisms are known (*mVOC database*, Lemfack et al. 2018) which are categorized into fatty acid derivatives, aromatic compounds, nitrogen-containing compounds, volatiles sulfur compounds, terpenoids and others such as halogenated compounds, and metalloid compounds (summarized in Schulz and Dickschat 2007).

Schenkel et al. (2015) used the *mVOC database* to quantify and compare compound classes released from microbes and plant roots. It is interesting to note that the same compound classes are found in both headspaces, while the quantitative distribution is different in these organisms. It is presently not studied in detail whether the appearance of identical or similar compounds in both kingdoms are due to evolutionary convergence (in analogy as described by Beran et al. 2019) or due to horizontal gene transfer (Jia et al. 2019). Fatty acids and respective derivatives such as alkanes, alkenes, aldehydes, ketones, alcohols, as well as ethers and esters are most likely products of incomplete oxidations of the primary metabolism. However, typical secondary metabolites are found in the groups of terpenes, aromatic compounds, furans, and S- and N-containing compounds.

Terpene Biosynthesis Terpenoids are the most diverse class of natural products, 80,000 compounds are estimated to be biosynthesized (Christianson 2017). Monoterpene (C10) and sesquiterpene (C15) compounds are most relevant as volatile organic compounds. Terpene synthases catalyze the most complex chemical reactions in biology since the carbon atoms of the substrates undergo complicated changes in bonding and hybridization during single enzyme catalyzed cyclization reactions. The classical substrates of terpene synthases are geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP) which are synthesized from C5 building blocks (isopentenyl pyrophosphate IPP and dimethylallyl pyrophosphate DMAPP). The latter derive either from the mevalonate

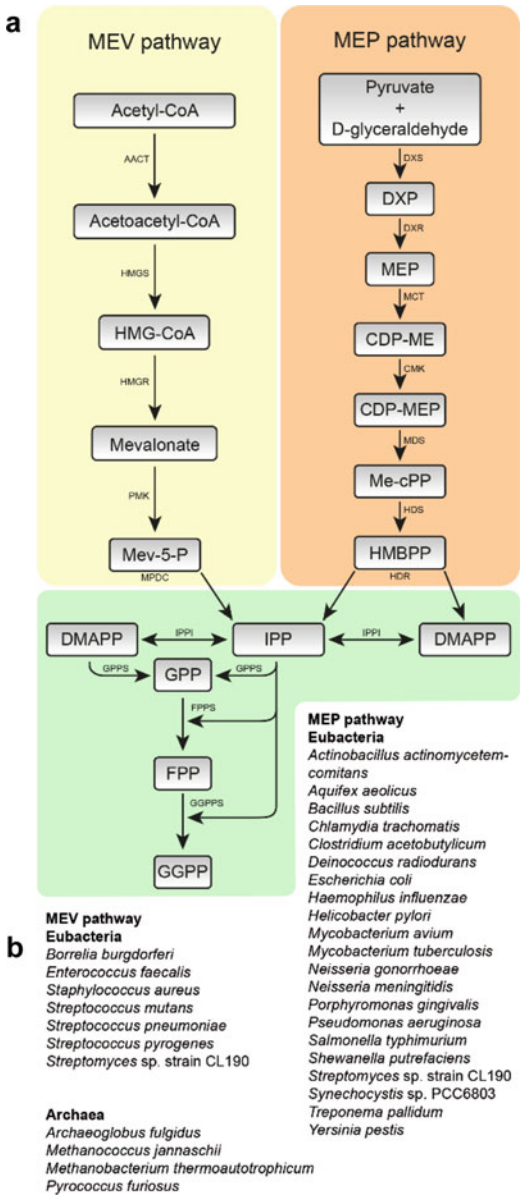

Fig. 1.10 Terpene biosynthesis. (**a**) Mevalonate and MEP pathway for IPP biosynthesis. (**b**) Examples of bacteria using one or the other pathway to produce IPP

or MEP pathway present in the different bacterial species (examples given in Fig. 1.10). Many terpene synthases are very specific and accept only one substrate (single substrate enzymes), while multisubstrate enzymes react with more than one prenyl pyrophosphate. However, the most outstanding and common feature of terpene synthases is their ability to produce multiple products from one substrate

(multiproduct enzymes). Often the products are released in defined ratios indicating that precise pathways of biosynthesis are underlying.

Beside the canonical substrates of terpene synthases GPP and FPP and their respective isomers (*E,E; Z,Z; E,Z*), NPP (neryl pyrophosphate) was described as a substrate (Jia et al. 2018; Sun et al. 2016), and it was shown that methyl-GPP was the substrate for methylisoborneol biosynthesis in *Streptomyces coelicolor* (Komatsu et al. 2008; Wang and Cane 2008). The latter opened a new route of structural diversity due to the fact that C11 compounds are also substrates for terpene synthases (Kschowak et al. 2018). Such methylation reactions also occur with IPP in *Streptomyces* species resulting in C6 substrates (Drummond et al. 2019) and FPP as shown in *Serratia plymuthica* 4Rx13 (Fig. 1.11a; von Reuss et al. 2018). The latter is particularly interesting because the FPP-methyltransferase (FPPMT) not only methylates the C15 FPP to a C16 compound but also performs a cyclisation reaction which is unique for methyltransferases. The product of the FPPMT is presodorifen pyrophosphate and expands the repertoire of non-canonical substrates of terpene synthases uniquely. So far, the methylation and cyclization reactions of IPP, GPP, and FPP (Fig. 1.11b) were only found in the bacterial metabolism.

Ca. 100 volatile monoterpenes and sesquiterpenes of bacterial origin were summarized by Schulz and Dickschat (2007). Several genome mining approaches were performed (Cane and Ikeda 2012; Yamada et al. 2012), however, up to now only 63 bacterial terpene synthases, primarily sesquiterpene synthases from *Streptomyces* species, have been isolated (Dickschat 2016). The architecture of bacterial terpene synthases is distinct compared to respective plant enzymes which typically are built of alpha, beta, and gamma domains, while respective bacterial enzymes are comprised of either single or double alpha domains or beta-gamma domains. The "alpha-only" type is most prevalent in bacteria. Furthermore, the characteristic aspartate-rich motive of plant terpene synthases (DDxxD) is slightly altered to DDxxxD in bacteria (Jia et al. 2018, 2019).

Aromatic Compounds

The basal biosynthetic pathway for aromatic secondary metabolites in plants and bacteria is the shikimate pathway whose primary products are the aromatic amino acids tyrosine, phenylalanine, and tryptophane. While the phenylpropane biosynthesis is very widespread and common in plants, the aromatic compound biosynthesis is (to date) not universally observed and well-studied in bacteria. However, compounds like 2-phenylethanol, phenol, benzyl alcohol, methyl benzoate, benzaldehyde, acetophenone, and closely related compounds were shown to be released from several bacteria (summarized by Schulz and Dickschat 2007, *mVOC database* Lemfack et al. 2018). Two alternative pathways (phenylalanine lyase pathway and phenylpyruvate–phenylacetate–phenylglycolate pathway, Figs. 1.12 and 1.13, respectively) are known to be involved in the biosynthesis of the above-mentioned compounds. However, it is difficult to distinguish these two pathways by feeding experiments with isotope labelled intermediates, and in many cases the bacteria developed individually altered enzymatic reactions supporting and expanding these general pathways. While benzaldehyde can be biosynthesized via both pathways, it

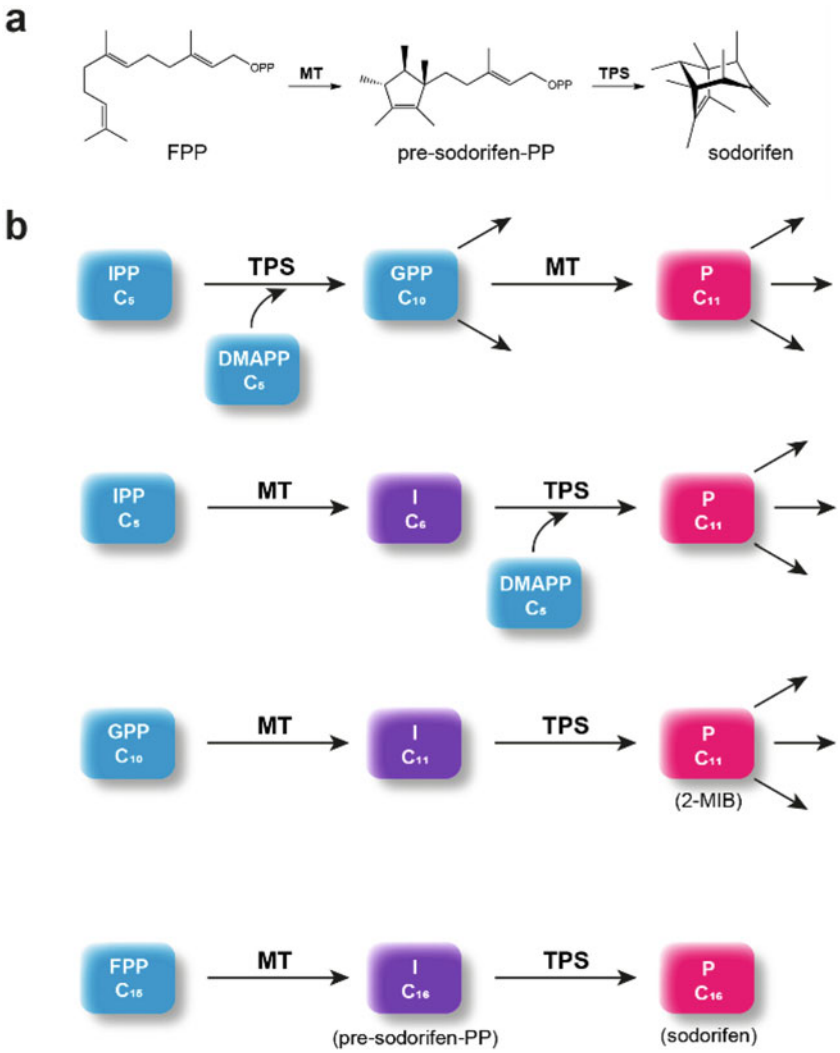

Fig. 1.11 (**a**) Biosynthesis of the extraordinary biosynthesis of sodorifen by *S. plymuthica* (after von Reuss et al. 2018). In a first step a methyltransferase methylates and forms a 5-carbon ring from the canonical substrate FPP; presodorifen is subsequently rearranged to sodorifen by a terpene synthase. (**b**) Schematic presentation of the canonical and non-canonical terpene biosynthesis to reveal methylated terpene products

seems straightforward that many bacteria synthesize β-phenylethanol via phenylacetaldehyde.

Pyrazines are a special class of volatile heterocyclic compounds that are often produced by many bacteria during fermentation processes (*mVOC database* Lemfack et al. 2018).

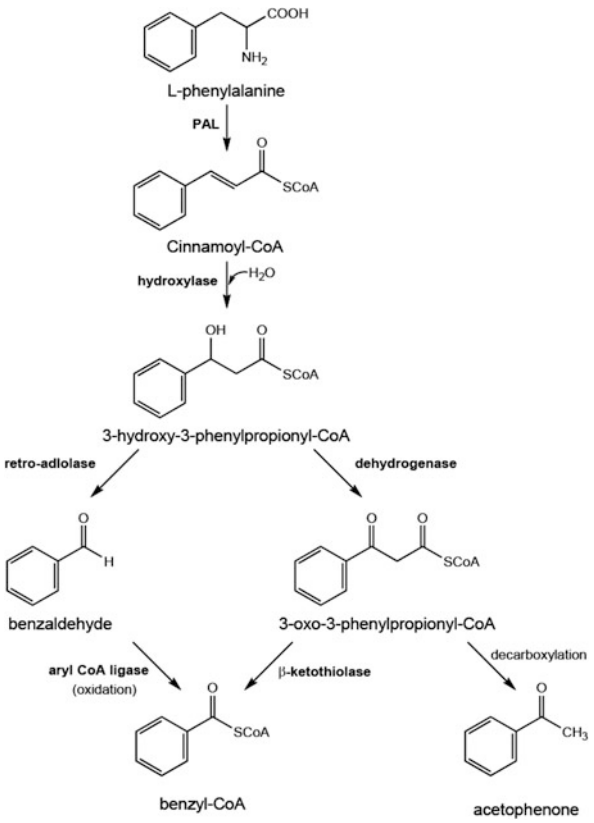

Fig. 1.12 Biosynthesis of aromatic compounds via phenylalanine lyase pathway

They exhibit strong odor properties and are therefore used as flavoring compounds from the aroma industry. Particularly widespread are pyrazines with one to four methyl or ethyl groups (simple alkylated pyrazines). Caution has to be taken because these pyrazines may also originate from cultivation media or are formed during heating or autoclaving. Pyrazines with longer side chains are less often found as bacterial volatiles. To clarify the biosynthesis of pyrazines, more research is needed. At present, one non-enzymatic pathway via amination of acyloins is postulated for the biosynthesis of simple pyrazines. This biosynthesis results in the formation of aminocarbonyl compounds. Condensation of two aminocarbonyl compounds leads to unstable dihydropyrazines (Fig. 1.14) which are easily oxidized to pyrazines. Higher alkylpyrazines require enzymatic reactions and derive from amino acids (Fig. 1.15). The methoxy methyl-group originates from *S*-adenosyl methionine.

Indole is a very prominent volatile biosynthesized by *E. coli*, other *Enterobacteriaceae* such as *Klebsiella* and *Enterobacter*, and other bacteria as well (e.g., *Loktanella*). It derives from a one-step reaction of the enzyme tryptophanase of the aromatic amino acid tryptophane (Fig. 1.16). The malodourous

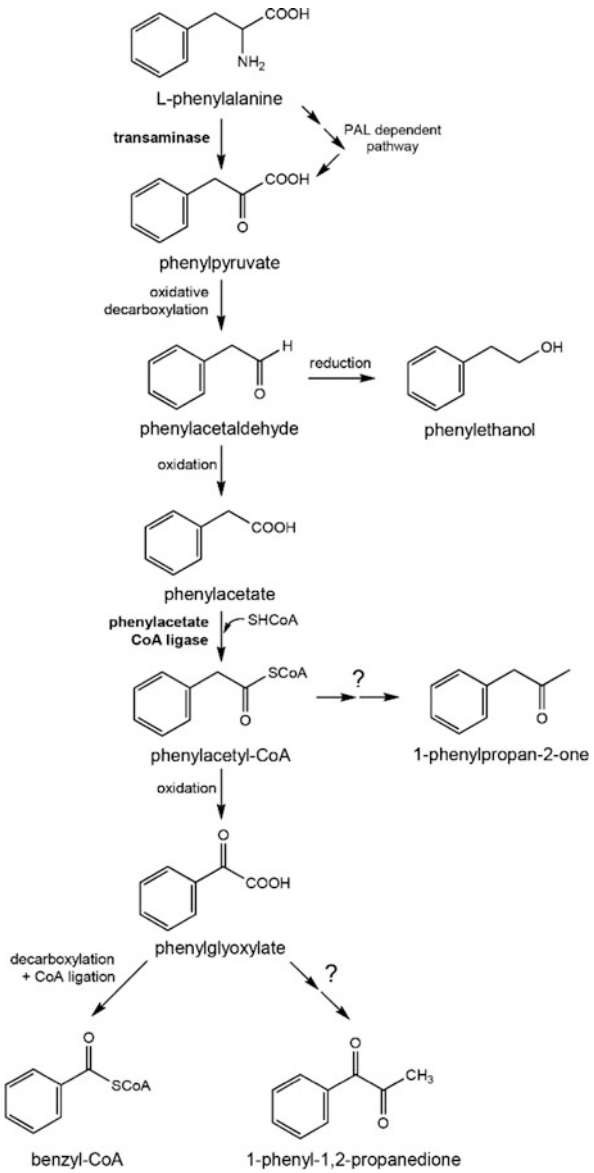

Fig. 1.13 Biosynthesis of aromatic compounds via phenylpyruvate–phenylacetate–phenylglycolate pathway

skatole also derives from tryptophane; it is released from *Calothrix* and biosynthesized via indole acetic acid (IAA) which is a well-known phytohormone.

N-Compounds

Ammonia is an inorganic, highly volatile compound and produced by many bacteria, including *Enterobacter*, *Serratia*, *Klebsiella*, *Staphylococcus*, *Micrococcus*, and *Bacillus* by degradation of amino acids, by nitrite ammonification, by

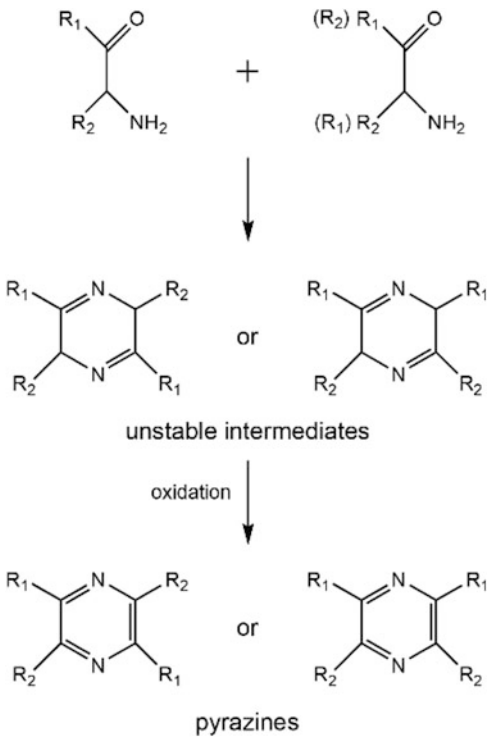

Fig. 1.14 Biosynthesis of simple pyrazines with methyl or ethyl side chains

urease-mediated hydrolytic degradation of urea, and by decarboxylation of amino acids (summarized in Piechulla et al. 2017). Biogenic amines such as trimethylamine, 2-methylpropylamine, 2-methylbutylamine, 3-methylbutylamine, cyclohexylamine, and phenylethylamine are also often found in the headspace of bacteria.

Biogenic amines can be converted by aminooxidases to respective aldehydes which furthermore can react with biogenic amines to produce imines.

S-Compounds

Dimethyl disulfide (DMDS) and dimethyl trisulfide (DMTS) are the most prominent volatile sulfur organic compounds released from bacteria (*mVOC database*, Lemfack et al. 2018). Three major pathways are known for their biosynthesis (Fig. 1.17). Marine bacteria (Alpha-, Beta-, Gamma-, Deltaproteobacteria) mainly use the dimethylsulfoniopropionate (DMSP) which is produced by algae in high amounts from L-methionine and is therefore prevalent in oceans (Fig. 1.17a). Depending on the bacterial species and its genetic repertoire, DMSP is converted to acrylate and DMS via CoA ester and acyl-CoA transferase, lytically cleaved or degraded by an unknown mechanism of DMSP lyase. Alternatively, DMSP can also be degraded on the demethylation pathway to 3-(methylmercapto) propionic acid by the DMSP demethylase and further to methanethiol and acrylate by an unknown

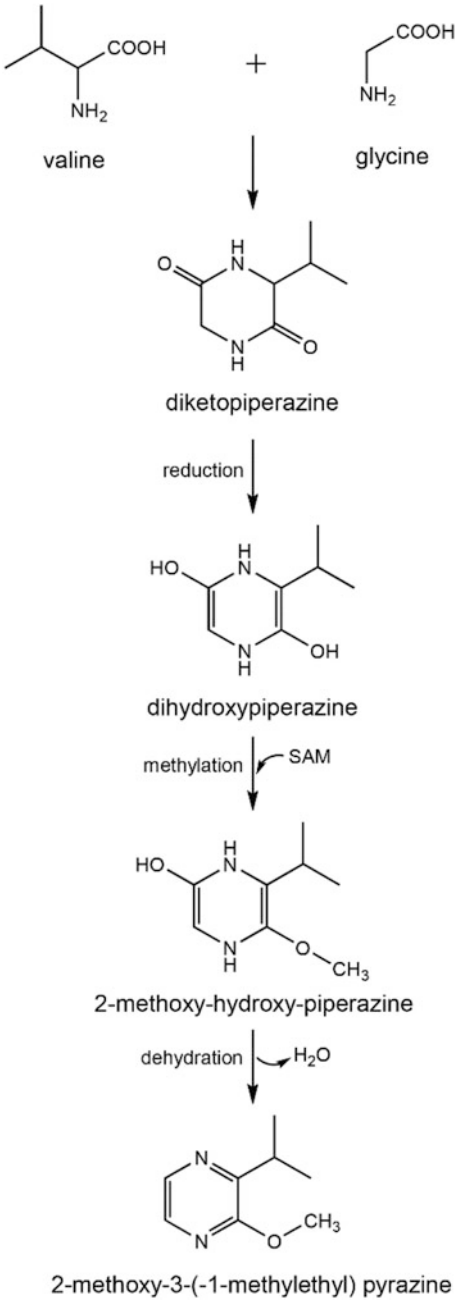

Fig. 1.15 Biosynthesis of higher alkylpyrazines

enzyme (Dickschat et al. 2010). In freshwater habitats, bacteria produce methanethiol and DMS through methylation of inorganic sulfide (Fig. 1.17b). In a

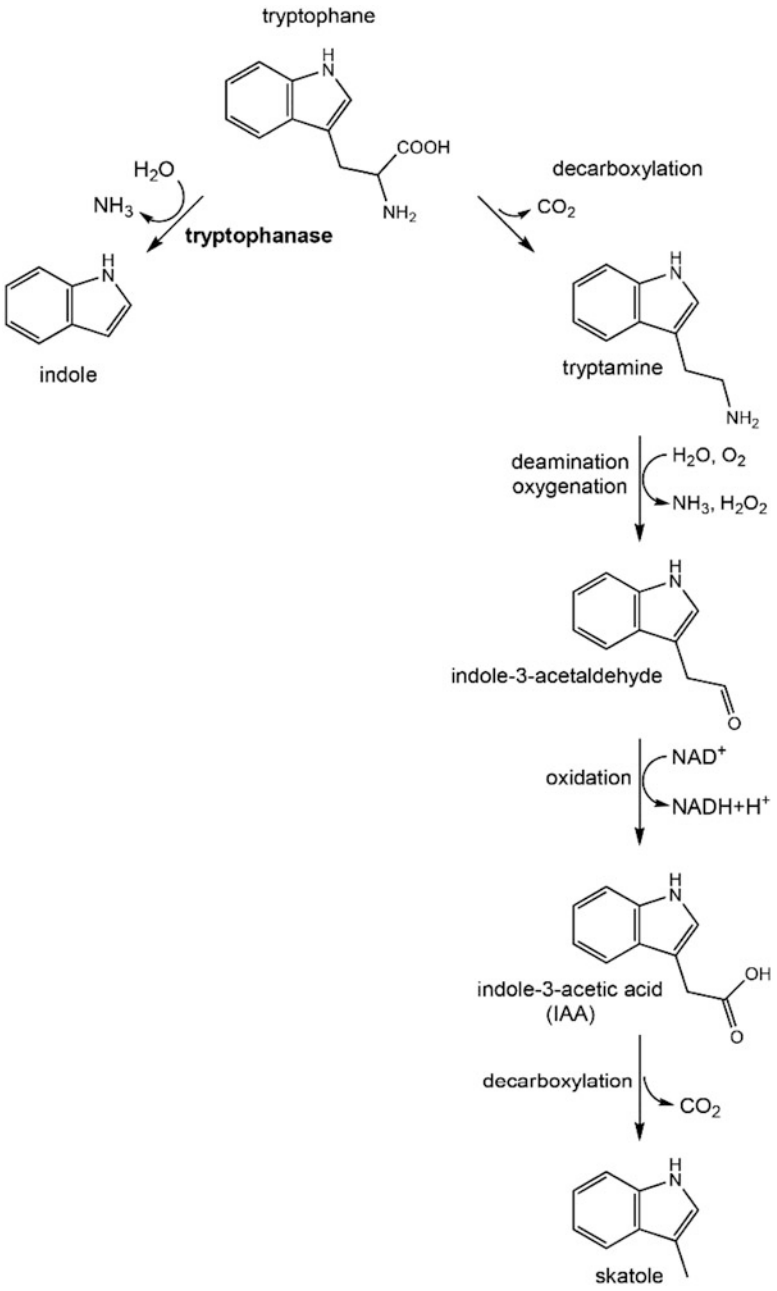

Fig. 1.16 Biosyntheses of indole and skatole derived from tryptophane

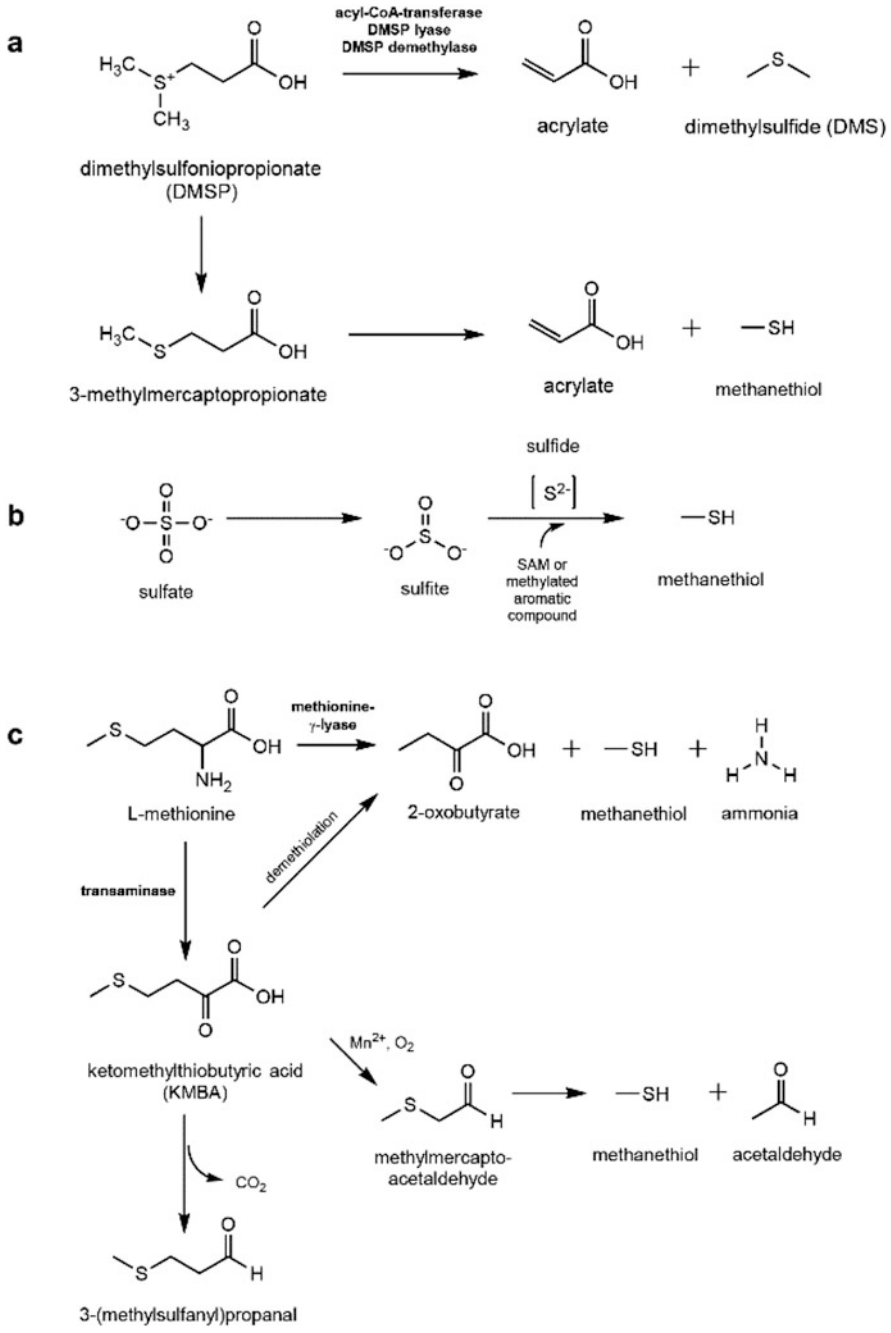

Fig. 1.17 Three biosynthetic pathways for volatile sulfur compounds in bacteria. (**a**) Dimethylsulfoniopropionate (DMSP) from bacteria of marine or estuarine habitats is converted into acrylate and dimethyl sulfide (DMS). (**b**) Bacteria in freshwater mainly produce methanethiol from sulfate. (**c**) L-Methionine is degraded by methionine lyase to ammonia, 2-oxobutyrate, and methanethiol or transaminated to ketomethylthiobutyric acid (KMBA). The latter may be converted by a proposed demethiolase to 2-oxobutyrate and methanethiol. KMBA can be chemically (Mn^{2+}, O_2) degraded to methyl mercapto acetaldehyde and subsequently converted to methanethiol and acetaldehyde, or to 3-(methylsulfanyl) propanal and several derivatives. (**d**) Methanethiol is a precursor for DMS, dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS)

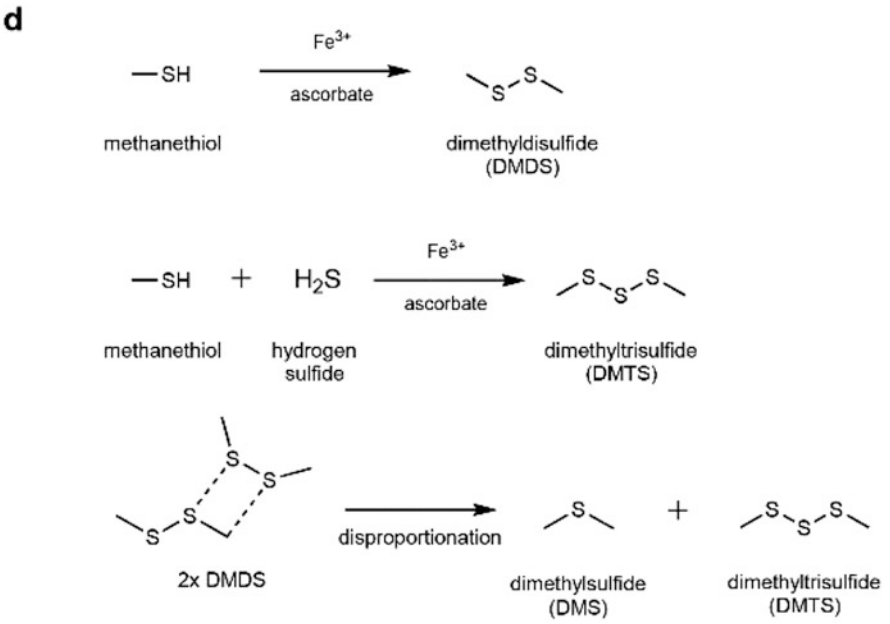

Fig. 1.17 (continued)

first step, sulfate is reduced to sulfite by sulfite reductase, which is an evolutionary old reaction/enzyme also present in archaea.

Methanethiol and DMS are then either produced by a SAM dependent methyltransferase or via methoxylated aromatic compounds (e.g. in, *Halophaga foetida, Sporobacter termitidis, Sporobacterium olearium;* and *Parasporobacetrium paucivorans*, respectively). Finally, L-methionine is the major source for volatile sulfur compounds of dairy product producing bacteria such as *Brevibacterium, Corynebacterium, Staphylococcus, Lactococcus,* and *Lactobacillus*. These bacteria produce a wide array of sulfur compounds biosynthesized via two methionine degradation pathways, (1) direct cleavage of methionine and (2) transamination to ketomethylthiobutyric acid (KMBA) and subsequent reductive demethiolation or decarboxylation (Fig. 1.17c). In the direct cleavage pathway methionine lyase produces ammonia, 2-oxobutyrate and methanethiol. The second pathway is initiated by a transaminase reaction and demethiolase to reveal 2-oxobutyrate and methanethiol, or alternatively KMBA is converted to 3-(methylsulfanyl) propanal, which is the precursor for subsequent reduction and oxidation reactions forming sulfur compound derivatives. Methanethiol can be converted by rapid autooxidation to DMDS, or by reaction with H_2S to DMTS. Alternatively, two molecules of DMDS can be transformed by disproportionation to DMS and DMTS (Fig. 1.17d).

Taken together, investigating more bacterial strains, even those from less representative phyla, classes, families, or genera, could help to discover new interesting

and bioactive natural products, especially those derived from the secondary metabolism. These could, in turn, be used for new applications since mVOCs are seen as new frontier in bioprospecting and can be applied as eco-friendly alternatives to synthetic compounds for biotechnological applications.

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Chapter 2
Bioactive Bacterial Organic Volatiles: An Overview and Critical Comments

Birgit Piechulla, Marie Chantal Lemfack, and Nancy Magnus

Abstract Microorganisms release numerous volatile organic compounds (VOCs) among which at the present state of the art ca. 10% have been shown to be biologically active. Fourteen compounds that target different organisms are considered unspecific. Out of the ca. 120 discrete VOCs the 29, 31, 12, 22 compounds affect either plants, fungi, bacteria, or invertebrates, respectively, or were so far only tested in the respective interorganismal interaction. They are tentatively considered specific and are most likely candidate compounds for future applications.

This chapter also raises critical aspects of dual co-culture test systems, regarding the consideration of inorganic and organic volatiles or single and complex VOC mixtures, single and multi-species approaches which share more similarity to the natural habitat and will provide a more holistic understanding of the volatile-mediated interactions in a given ecological niche.

Keywords Bacterial volatile organic compounds · Volatile-mediated interorganismal interaction · Bioactive VOC · Co-culture · Sodorifen · *mVOC database*

2.1 Introduction

Microorganisms are universally present in nature and are able to colonize even extreme habitats. In the last decade, the microbiomes of various habitats such as in and on invertebrates, animals, humans (e.g., guts or skins), or of the phyllosphere and rhizosphere of plants were systematically analyzed. Besides describing the appearance and the phylogenetic relationships of bacteria in microbiomes, it is also important to consider the metabolic capabilities of these microorganisms. For instance, the knowledge regarding the uptake and metabolization of organic

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compounds is indispensable because respective intermediates and/or end products may serve different biological functions in the host organism (e.g., play a role during digestion) or in the ecological niche (e.g., they are important components of the natural recycling processes of litter). Microorganisms can survive in high as well as in scarce nutritional conditions by adapting their metabolisms correspondingly. To maintain their viability, they are extremely flexible in metabolic and physiological adjustments, e.g., it is well documented that under competitive circumstances microbes produce secondary/specialized metabolites. Many isolated microorganisms are able to produce high molecular weight effective (=bioactive) compounds some of which are used as antibiotics in human health care. However, small compounds of 300 Da or less were overlooked in the past. Research of these microbial compounds (microbial volatile organic compounds, mVOCs) experienced a renaissance when Ryu et al. (2003) and others (summarized in Kai et al. 2009) published plant growth promoting and plant pathogen inhibiting effects of these mVOCs. Meanwhile, a large number of publications with similar results appeared, and the initial work was extended by recording the reactions to mVOCs of several plants and fungi (Effmert et al. 2012; Piechulla et al. 2017).

Based on a literature screen for known mVOCs the *mVOC database* was established (Lemfack et al. 2014, 2018). Presently, ca. 2000 mVOCs are registered from ca. 1000 fungi and bacteria. The *mVOC database* can already be regarded as substantial, but many more compounds are expected to be found in the future, considering the fact that so far only 10^3 microorganisms have been investigated although 10^{16} bacterial species are extrapolated to exist on earth (Farré-Armengol et al. 2016). The contribution of Lemfack et al. in this book gives a comprehensive overview about the bacterial phylogeny and the bacterial primary and secondary metabolisms. The structural diversity of the VOCs released from bacteria is described by Schulz et al. (2020). The extraordinary potential of microorganisms synthesizing unique and outstanding VOCs is exemplified by sodorifen, an unusual compound emitted solely by *Serratia plymuthica* isolates (Kai et al. 2010; von Reuss et al. 2010). The basis of the structural plethora resides in the (individual) microbial metabolic capacity and additionally reflects the substrate usage and its availability (Lemfack et al. 2020; Fiddaman and Rossall 1994; Zareian et al. 2018). It is important to note that substrate availability in the lab and in nature are considerably different, therefore, VOC profiles obtained from bacteria grown under lab conditions are most likely very different to VOC profiles of microorganisms living in the field/nature/ecological niche. Since it is not possible to investigate *all* growth conditions, the all-embracing potential of compound biosynthesis of given bacteria remains unknown. Moreover, it is well known that microbial VOC production has been exploited for human preferences, for example, food such as cheese, wine, beer, and yoghurt emit aromas acceptable for human taste and nose.

Besides their importance in food aromas, microbial volatiles are also used in various other applications. Attention was given to mVOCs as indicators for contaminants and pollutants with potential consequences for human health (Korpi et al. 2009). mVOCs used in medicine, agriculture, and biotechnological applications were recently addressed and summarized by Piechulla and Lemfack (2016) and

are substantiated by Elmassry and Farag (2020), Kusstatscher et al. (2020). The present demand for new bioactive compounds for different applications is large, since, e.g., many antibiotics of the past generation cannot be used anymore due to resistance phenomena, or the use of herbicides, insecticides, nematicides, etc. is excluded due to new safety regulations. Therefore, bioprospecting among thousands of VOCs produced by microorganisms may result in some new molecules with specific bioactivities matching the demanded requirements. The properties of the small molecules, e.g., low boiling point, high vapor pressure, and lipophilic character, may also direct towards new application formats. The determination of bioactivity profiles of complex mVOC blends of bacteria, of discrete mVOCs or defined mixtures of mVOCs is indispensable to support future hopes for establishing biologically safe strategies.

In the past decade, the potential of mVOCs was already indicated. The effects of mVOCs were explored in various experimental co-cultivation set-ups with plants (Chung et al. 2016; Kai et al. 2016), insects (Davis et al. 2013), soil fauna, bacteria, and fungi (De Vrieze et al. 2015; Schmidt et al. 2015; Werner et al. 2016). Sometimes different results were observed, most likely because the interactions of the microorganisms with the receiver organisms are complex and mVOCs may act at multitrophic levels as multitrophic signals. Since the ecological systems are not always well studied or defined, interactions are difficult to assign to distinct targets (Piechulla et al. 2017). Moreover, it is critical whether the receiver organism perceives cues from a mixture of mVOCs or from a discrete (=single, =individual) compound. To date, most results were obtained from co-cultivation experiments, with the consequence that the receiver organism was fumigated by quantitatively and qualitatively complex and dynamically altered volatile mixtures, including organic as well as inorganic volatiles. The rational for using these co-cultivations is that in nature bacteria appear in the same habitat as the target organism and all or several volatiles of the emitter might affect the receiver similar as under natural conditions, and vice versa. However, the dual co-cultivations, as performed in the lab, have several drawbacks: (1) in nature, large bacterial communities rather than only one or two bacterial species colonize the habitat, (2) bacterial densities in Petri dishes easily reach 10^{11} CFU, which in average is much higher than under natural conditions, (3) in nature, the bacterial metabolism depends on the different and variable exudates or litter, while in the lab usually one major, easy accessible carbon source (often glucose) is provided, and (4) in the lab, bacteria live under comfortable environmental and nutrient conditions in contrast to the harsh and often substrate limiting conditions in nature. Since the mVOC-mediated bacteria-target interactions are very complex, it is presently impossible to transfer this complexity, flexibility, variability, and dynamic to laboratory experiments. It will be a challenge for the future to design laboratory experiments that simulate the natural reality, in order to unravel the genuine ecological and biological roles of bacterial volatiles. A present strategy is to perform reductionistic approaches, e.g., to expose the target organisms to single bioactive VOCs or defined mixtures of known quantity and ratios deduced from the natural sources.

To get an overview about presently known bioactive discrete bacterial VOCs the *mVOC database* was screened. Fungal VOCs were not included in this survey. Bacterial VOCs (bacVOCs), applied to the different receiver organisms (plants, fungi, bacteria, and invertebrates), are summarized in Tables 2.1, 2.2, 2.3, and 2.4 (Fig. 2.1). The analyses are based on 545 respective entries and are separately discussed in the following chapters. Surveying the extracted data, it becomes obvious that systematic experimentations are lacking. Therefore, the present abstractions of the observations can only indicate trends, while solid and fundamental insights remain elusive. More systematic approaches are needed in the future to corroborate the biological and ecological impact of discrete bacVOCs.

2.2 Bioactive Bacterial VOCs Affecting Plants

Forty-seven different bacterial VOCs were so far tested on plants (Table 2.1), comprising 3.6% of the ca. 2000 known mVOCs (Lemfack et al. 2018). The most frequently tested bacVOCs are 1-hexanol, 2-phenylethanol, 2,3-butanediol, acetoin, dimethyl disulfide, and indole. 2,3-Butanediol and the related compound acetoin (3-hydroxy-2-butanone) were shown to promote plant growth (Ryu et al. 2003, 2004). These compounds affect several different plant phenotypes, such as induction of systemic resistance (ISR), PR gene activation, improvement of drought survival, and stomata closure and aperture. The sulfur containing compound dimethyl disulfide alters phenomena ranging from plant growth reduction, ISR, seed germination reduction, but also contradictory results such as seedling and plant growth promotions were observed. These contrasting results are most likely due to the different doses applied ("*Dosis sola venenum facit*"). 2-Phenylethanol is an aromatic volatile, characterized by a rose-like odor and its antimicrobial potential through the ability to alter properties of the plasma membrane (Etschmann et al. 2002). Moreover, 2-phenylethanol fumigated in bipartite Petri dishes resulted in dramatic growth inhibition of *A. thaliana* seedlings (Wenke et al. 2012a, 2018). Indole is a well-known VOC from Enterobacteriaceae but is also released from other genera. It is a bioactive compound; in plants ethylene production is blocked and oxidative burst occurs. Further examples can be looked up in Table 2.1.

So far, almost all studies glanced at rather coarse morphological and phenotypical changes (e.g., biomass alteration, root length and architecture, seed germination) in plants (Table 2.1). It is quite surprising that the bacVOC fumigations reveal such dramatic and easily visible alterations in the plant, while it seems more likely that the plants would react by (invisible) changes expressed at the biochemical or molecular level. Pentadecane and hexanol were shown to alter MAMP (microbial associated molecular pattern) responses and tridecane altered primed gene expression. To understand the biological function of discrete bacVOCs many more investigations have to address molecular targets, for example, by performing transcriptome or metabolome analyses. Furthermore, subtle (not only coarse) responses have to be recorded and distinct targets have to be considered.

Table 2.1 Bacterial VOCs affecting plants

Pubchem_id	Compound	Emitter	Receiver_plant	References
8103	1-Hexanol	*Burkholderia* sp.	*Arabidopsis thaliana*, MAMP response, flg-22 induced ethylene production; flg22 induced ROS production inhibited, 1 ng to 1 mg	Blom et al. (2011)
8103	1-Hexanol	*Cellulomonas uda*	*Arabidopsis thaliana*, MAMP response, flg-22 induced ethylene production; flg22 induced ROS production inhibited, 1 ng to 1 mg	Blom et al. (2011)
8103	1-Hexanol	*Chromobacterium violaceum* CVO	*Arabidopsis thaliana*, MAMP response, flg-22 induced ethylene production; flg22 induced ROS production inhibited, 1 ng to 1 mg	Blom et al. (2011)
8103	1-Hexanol	*Escherichia coli* OP50	*Arabidopsis thaliana*, MAMP response, flg-22 induced ethylene production; flg22 induced ROS production inhibited, 1 ng to 1 mg	Blom et al. (2011)
8103	1-Hexanol	*Paraburkholderia phytofirmans*	*Arabidopsis thaliana*, growth promotion, salinity tolerance, 100 ng	Ledger et al. (2016)
8103	1-Hexanol	*Pseudomonas simiae*	*Glycine max*, seed germination inhibition, 50–100 μg	Vaishnav et al. (2016)
8103	1-Hexanol	*Pseudomonas* sp. *Serratia* sp.	*Arabidopsis thaliana*, MAMP response, flg-22 induced ethylene production; flg22 induced ROS production inhibited, 1 ng to 1 mg	Blom et al. (2011)
522224	1-Methyl-thio-3-pentanone	*Burkholderia ambifaria*	*Arabidopsis thaliana*, reduction of plant biomass, no necrosis, no chlorosis, 1 ng, 1 μg, 1 mg	Groenhagen et al. (2013)
6276	1-Pentanol	*Bacillus* sp.	Pathogen infection reduced in pepper	Chung et al. 2016
13190	1-Undecene	*Pseudomonas tolaasii*	Broccoli and lettuce seed germination inhibition, 50–500 μg	Lo Cantore et al. (2015)
–	13-Tetradecadiene-1-ol	*Pseudomonas fluorescens* SS101	*Nicotiana tabacum* cv, Xanthi-nc, plant growth, fresh weight, 5 ng, 50 ng, 500 ng	Park et al. (2015)
6569	2-Butanone	*Bacillus amyloliquefaciens* IN937a	ISR, protection against *Pseudomonas syringae*, induction oxylipin biosynthesis	Song and Ryu (2013)

(continued)

Table 2.1 (continued)

Pubchem_id	Compound	Emitter	Receiver_plant	References
6569	2-Butanone	*Bacillus subtilis* GB03	ISR, protection against *Pseudomonas syringae*, induction oxylipin biosynthesis	Song and Ryu (2013)
6569	2-Butanone	*Pseudomonas fluorescens* SS101	*Nicotiana tabacum* cv, Xanthi-nc, plant growth, fresh weight, 5 ng, 50 ng, 500 ng	Park et al. (2015)
140334	2-Methyl-*n*-1-tridecene	*Pseudomonas fluorescens* SS101	*Nicotiana tabacum* cv, Xanthi-nc, plant growth, fresh weight, 5 ng, 50 ng, 500 ng	Park et al. (2015)
19602	2-Pentylfuran	*Bacillus megaterium* XTBG34	*Arabidopsis thaliana* growth promotion	Zou et al. (2010)
69012607	2-Phenyl ethyl acetate	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 μM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
6054	2-Phenylethanol	*Serratia plymuthica* HRO C48	Arabidopsis thaliana, growth reduction, 16.8 and 168 μmol	Wenke et al. (2012a), Wenke et al. 2012b)
6054	2-Phenylethanol	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 μM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
6054	2-Phenylethanol	Rhizosphere and phyllosphere microbiota	*Agave tequila*, *Agave salmiana*, 5 μM, 50 μM, 0.5–2 fold change, main leaf length, root thickness, root length	Camarena-Pozos et al. (2019)
11622	2-Tridecanone	*Sinorhizobium meliloti*	Alfalfa, *Medicago*, reduction of nodulation, 5 μM, 25 μM	López-Lara et al. (2018)
8163	2-Undecanone	*Paraburkholderia phytofirmans*	*Arabidopsis thaliana*, growth promotion, salinity tolerance, 100 ng	Ledger et al. (2016)
262	2,3-Butanediol	*Enterobacter aerogenes*	*Zea mays* resistant against Northern corn leaf blight fungus *Setosphaeria turcica*	D'Alessandro et al. (2014)

262	2,3-Butanediol	*Bacillus amyloliquefaciens* L3	*Arabidopsis thaliana* Col 0, 500 µg	Wu et al. (2019)
262	2,3-Butanediol	*Bacillus badius* M12	Apple pieces, polyphenol oxidase	Gopinath et al. (2015)
16547	2,4-Diacetylphloroglucinol	*Pseudomonas* sp.	Defense of plant roots	Summarized in Weller et al. (2007)
262	2*R*, 3*R*-butanediol	*Pseudomonas chlororaphis* O6	Tobacco, enhanced growth, ISR against *Erwinia carotovora*, but not against *Pseudomonas syringae*	Han et al. (2006), Ryu et al. (2003)
8064	2*R*,3*R* butanediol,2*S*,3*S* butanediol	*Bacillus amyloliquefaciens* IN937a	*Arabidopsis thaliana* resistance against *Erwinia carotovora*, 10 ng to 1000 mg; tobacco, PR genes and defense priming upregulated	Ryu et al. (2003, 2004), Han et al. (2006)
8064	2*R*,3*R* butanediol,2*S*,3*S* butanediol	*Bacillus subtilis* GB03	*Arabidopsis thaliana* resistance against *Erwinia carotovora*, 10 ng to 1000 mg; tobacco, PR genes and defense priming upregulated	Ryu et al. (2003, 2004), Han et al. (2006)
10448	3-(methylthio)-1-propanol	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 µM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
11509	3-Hexanone	*Burkholderia ambifaria*	*Arabidopsis thaliana*, increase of plant biomass, 1 ng, 1 µg, 1 mg	Groenhagen et al. 2013
31260	3-Methyl-1-butanol	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 µM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
31260	3-Methyl-1-butanol	Rhizosphere and phyllosphere microbiota	*Agave tequila*, *Agave salmiana*, 5 µM, 50 µM, 0.5–2 fold change, main leaf length, root thickness, root length	Camarena-Pozos et al. (2019)
31260	3-Methyl-butanol	*Paraburkholderia phytofirmans*	*Arabidopsis thaliana*, growth promotion, salinity tolerance, 100 ng	Ledger et al. (2016)

(continued)

Table 2.1 (continued)

Pubchem_id	Compound	Emitter	Receiver_plant	References
11428	3-Pentanol	Many bacteria	*Capsicum annum* ISR, resistance against *Xanthomonas axonopodis* and cucumber mosaic virus; SA and JA signaling; field experiment	Choi et al. (2014)
24901674	4-Methyl-thio-2-butanone	*Burkholderia ambifaria*	*Arabidopsis thaliana*, reduction of plant biomass, no necrosis, no chlorosis, 1 ng, 1 µg, 1 mg	Groenhagen et al. (2013)
76738	4-Nitroguaiacol	*Pseudomonas simiae*	*Glycine max*, seed germination, 50–100 µg	Vaishnav et al. (2016)
10408	6,10,14-Trimethyl-2-pentadecanone	Rhizobacteria	*Arabidopsis thaliana*, root system architecture	Gutiérrez-Luna et al. (2010)
179	Acetoin	*B. megaterium* SA1	*Lactuca sativa*, root length and number of lateral roots increased, 0.1 µg to10 mg	Fincheira et al. (2016)
179	Acetoin	*B. pumilus* I-6	*Lactuca sativa*, root length and number of lateral roots increased, 0.1 µg to 10 mg	Fincheira et al. (2016)
179	Acetoin	*Bacillus amyloliquefaciens*	*Citrus*, reduction of post-harvest effects caused by *Penicillium digitatum*, *Penicillium italicum*, *Penicillium crustosum*	Arrebola et al. (2010)
179	Acetoin	*Bacillus amyloliquefaciens* L3	*Arabidopsis thaliana* Col 0, 1000 µg	Wu et al. (2019)
179	Acetoin	*Bacillus amyloliquefaciens* long C	*Lactuca sativa*, root length and number of lateral roots increased, 0.1 µg to 10 mg	Fincheira et al. (2016)
179	Acetoin	*Bacillus* sp. GB03	*Arabidopsis thaliana*, reduction of soft rot caused by *Erwinia carotovora*	Ryu et al. (2003, 2004)
179	Acetoin	*Bacillus subtilis*	*Citrus*, reduction of post-harvest effects caused by *Penicillium digitatum*, *Penicillium italicum*, *Penicillium crustosum*	Arrebola et al. (2010)
179	Acetoin	*Bacillus subtilis* FB17	*Arabidopsis thaliana*, ISR activated via SA/ET not JA pathway	Rudrappa et al. (2010)

179	Acetoin	*Bacillus amyloliqufaciens* IN937a	*Arabidopsis thaliana*, reduction of soft rot caused by *Erwinia carotovora*	Ryu et al. (2003, 2004)
7410	Acetophenone	*Burkholderia ambifaria*	*Arabidopsis thaliana*, increase of plant biomass, 1 ng, 1 μg, 1 mg	Groenhagen et al. (2013)
7410	Acetophenone	Rhizobacteria	Arabidopsis thaliana, root architecture	Gutiérrez-Luna et al. (2010)
240	Benzaldehyde	*Pseudomonas fluorescens* ALEB7B	*Atractylodes lancea*, increase of leaf net photosynthetic rate, superoxide dismutase, catalase, phenylalanine ammonia lyase, polyphenol oxidase	Zhou et al. (2016)
240	Benzaldehyde	Rhizobacteria	*Arabidopsis thaliana*, root architecture	Gutiérrez-Luna et al. (2010)
243	Benzoic acid	*Alcaligenes faecalis* JBC1294	*Arabidopsis thaliana*, shoot and root length and fresh weight increased, flesh weight increased, 0.21 mM	Bhattacharyya and Lee (2017)
7222	Benzothiazole	*Pseudomonas simiae*	*Glycine max*, seed germination, 50–100 μg	Vaishnav et al. (2016)
244	Benzyl alcohol	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 μM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
244	Benzyl alcohol	Rhizosphere and phyllosphere microbiota	*Agave tequila*, *Agave salmiana*, 5 μM, 50 μM, 0.5–2 fold change, main leaf length, root thickness, root length	Camarena-Pozos et al. (2019)
264	Butyric acid	*Alcaligenes faecalis* JBC1294	*Arabidopsis thaliana*, shoot and root length increased, fresh weight increased, 56.7 μM	Bhattacharyya and Lee (2017)
12232	Dimethyl disulfide	*Bacillus cereus* C1L	ISR in tobacco and maize against *Botrytis cinerea*, *Cochliobolus heterostrophus*, 0.1–10 mM	Huang et al. (2012)
12232	Dimethyl disulfide	*Bacillus* sp. B55	*Arabidopsis thaliana* growth promotion, 0–1 mM	Meldau et al. (2013)
12232	Dimethyl disulfide	*Burkholderia ambifaria*	*Arabidopsis thaliana*, increase of plant biomass, 1 ng, 1 μg, 1 mg	Groenhagen et al. (2013)

(continued)

Table 2.1 (continued)

Pubchem_id	Compound	Emitter	Receiver_plant	References
12232	Dimethyl disulfide	*Pseudomonas tolaasii*	Broccoli and lettuce seed germination inhibition, 2.5 μg; growth stimulation, 0.312 and 0.625 μg	Lo Cantore et al. (2015)
12232	Dimethyl disulfide	*Serratia plymuthica* 4Rx13	Arabidopsis thaliana, growth reduction, 0.5 μmol to 0.5 mmol	Kai et al. (2010)
19310	Dimethyl trisulfide	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 μM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
16221	Dimethylhexadecylamine	*Arthrobacter agilis* UMCV2	Sorghum, bicolor, 1.8 fold increase of shoot fresh weight, shoot length, chlorophyll concentration, lateral root number, lateral root concentration, 8 μM	Castulo-Rubio et al. (2015)
7165	Ethyl benzoate	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 μM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
7342	Ethyl isobutyrate	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 μM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
7945	Ethyl isovalerate	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 μM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
7945	Ethyl isovalerate	Rhizosphere and phyllosphere microbiota	*Agave tequila*, *Agave salmiana*, 5 μM, 50 μM, 0.5–2 fold change, main leaf length, root thickness, root length	Camarena-Pozos et al. (2019)

798	Indole	*Burkholderia* sp.	*Arabidopsis thaliana*, block of ethylene production, high indole: increase of oxidative burst, 1 ng to 1 mg	Blom et al. (2011)
798	Indole	*Cellulomonas uda*	*Arabidopsis thaliana*, block of ethylene production, high indole: increase of oxidative burst, 1 ng to 1 mg	Blom et al. (2011)
798	Indole	*Chromobacterium violaceum* CVO	*Arabidopsis thaliana*, block of ethylene production, high indole: increase of oxidative burst, 1 ng to 1 mg	Blom et al. (2011)
798	Indole	*Escherichia coli* OP50	*Arabidopsis thaliana*, block of ethylene production, high indole: increase of oxidative burst, 1 ng to 1 mg	Blom et al. (2011)
798	Indole	*Hyphomonas* sp.	*Chlorella vulgaris* OW01, growth promotion, 10–40 μM	Cho et al. (2019)
798	Indole	*Proteus vulgaris* JBLS202	Cabbage, 20–30% growth promotion by 0.63 μg	Yu and Lee (2013)
798	Indole	*Pseudomonas* sp. *Serratia* sp.	*Arabidopsis thaliana*, block of ethylene production, high indole: increase of oxidative burst, 1 ng to 1 mg	Blom et al. (2011)
798	Indole	Soil borne bacteria	*Arabidopsis thaliana*, root growth	Bailly et al. (2014)
798	Indole	*Stenotrophomonas rhizophila* ep10-p69	*Arabidopsis thaliana*, block of ethylene production, high indole: increase of oxidative burst, 1 ng to 1 mg	Blom et al. (2011)
31276	Isoamyl acetate	Rhizosphere and phyllosphere microbiota	*Arabidopsis thaliana*, 5, 10, 100, 500, 1000 μM, 1–3 fold change of fresh shoot biomass, total fresh biomass, fresh root biomass, chlorophyll content, primary root length, lateral root length, lateral root density, lateral root number, volatile mixtures also tested	Camarena-Pozos et al. (2019)
31276	Isoamyl acetate	Rhizosphere and phyllosphere microbiota	*Agave tequila*, *Agave salmiana*, 5 μM, 50 μM, 0.5–2 fold change, main leaf length, root thickness, root length	Camarena-Pozos et al. (2019)
878	Methanethiol	*Pseudomonas tolaasii*	Broccoli and lettuce seed germination inhibition, 50–500 μg	Lo Cantore et al. (2015)
7150	Methyl-benzoate	*Cladosporium*	*Arabidopsis thaliana*, suppression of symptoms, ISR, 1 μM to 100 mM	Naznin et al. (2014)
85258	*N*,*N*-dimethyl hexadecanamine	*Arthrobacter agilis*	*Medicago sativa*, growth, development, 8 mM; (related to QS signal)	Velázquez-Becerra et al. (2011)

(continued)

Table 2.1 (continued)

Pubchem_id	Compound	Emitter	Receiver_plant	References
12391	Pentadecane	*Burkholderia sacchari* LMG 19450	*Arabidopsis thaliana*, MAMP response, 1 ng to 1 mg	Blom et al. (2011)
12391	Pentadecane	*Serratia entomophilia* A1MO2	*Arabidopsis thaliana*, MAMP response, 1 ng to 1 mg	Blom et al. (2011)
24898186	Phenanzine	*Pseudomonas chlororaphis* O6	ISR	Han et al. (2006), Ryu et al. (2003)
1032	Propionic acid	*Alcaligenes faecalis* JBC1294	*Arabidopsis thaliana*, shoot and root length increased, fresh weight increased, 4.05 µM	Bhattacharyya and Lee (2017)
7047	Qunioline	*Pseudomonas simiae*	*Glycine max*, seed germination reduced, 50–100 µg	Vaishnav et al. (2016)
31291	Tetradecanal	Rhizobacteria	*Arabidopsis thaliana*, root system architecture	Gutiérrez-Luna et al. (2010)
25311	Tridecanal	Rhizobacteria	*Arabidopsis thaliana*, root system architecture	Gutiérrez-Luna et al. (2010)
12388	Tridecane	*Paenibacillus polymyxa* E681	*Arabidopsis thaliana*, ISR against *Pseudomonas syringae*, priming gene expression PR1, ChiB, VSP2, 0.1–10 mM	Lee et al. (2012), Han et al. (2006)
12388	Tridecane	*Pseudomonas simiae*	*Glycine max*, seed germination, 50–100 µg	Vaishnav et al. (2016)

Table 2.2 Bacterial VOCs affecting fungi

Pubchem_id	Compound	Emitter	Receiver_Fungi	References
8007	1-Butane amine	*Alcaligenaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 20–21 g/L; mycelium growth inhibition, 32–44 g/L	Zou et al. (2007)
8007	1-Butane amine	*Bacillales*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 20–21 g/L; mycelium growth inhibition, 32–44 g/L	Zou et al. (2007)
8007	1-Butane amine	*Micrococcaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 20–21 g/L; mycelium growth inhibition, 32–44 g/L	Zou et al. (2007)
8007	1-Butane amine	*Rhizobiaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 20–21 g/L; mycelium growth inhibition, 32–44 g/L	Zou et al. (2007)
8007	1-Butane amine	*Xanthomonadaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 20–21 g/L; mycelium growth inhibition, 32–44 g/L	Zou et al. (2007)
13381	1-Decene	*Alcaligenaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6 g/L; mycelium growth inhibition, 22–37 g/L	Zou et al. (2007)
13381	1-Decene	*Bacillales*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6 g/L; mycelium growth inhibition, 22–37 g/L	Zou et al. (2007)
13381	1-Decene	*Micrococcaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6 g/L; mycelium growth inhibition, 22–37 g/L	Zou et al. (2007)
13381	1-Decene	*Rhizobiaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6 g/L; mycelium growth inhibition, 22–37 g/L	Zou et al. (2007)

(continued)

Table 2.2 (continued)

Pubchem_id	Compound	Emitter	Receiver_Fungi	References
13381	1-Decene	*Xanthomonadaceae*	*Pochonia chlamydosporia, Paecilomyces lilacinus*, spore growth inhibition 6 g/L; mycelium growth inhibition, 22–37 g/L	Zou et al. (2007)
8183	1-Dodecene	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of sporangia germination (min. 30% inhibition)	De Vrieze et al. (2015)
18827	1-Octen-3-ol	*Bacillus pumilus* (S32)	*Phaeomoniella chlamydospora* antifungal activity, 41–410 mg/L	Haidar et al. (2016)
18827	1-Octen-3-ol	*Paenibacillus* sp (S19)	*Phaeomoniella chlamydospora* antifungal activity, 41–410 mg/L	Haidar et al. (2016)
7148	1-Phenyl propane-1-one	*Burkholderia ambifaria*	Growth reduction *Rhizoctonia solani*, no growth reduction *Fusarium solani*, 1 ng, 1 μg, 1 mg	Groenhagen et al. (2013)
13190	1-Undecene	*Pseudomonas*	*Phytophthora infestans* growth inhibition	Hunziker et al. (2015)
13190	1-Undecene	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*, dry mass reduction, 100 ppm	Briard et al. (2016)
13190	1-Undecene	*Pseudomonas tolaasii*	*Agaricus bisporus* discoloration, 2.5 μg; *Pleurotus ostreatus* mycelium growth inhibition, 2.5 μg	Lo Cantore et al. (2015)
996	1,3-Butadiene	*Bacillus amyloliquefaciens* VM42, *Bacillus velezensis* VM10, VM11, endophytes	Sclerotinia sclerotiorum, 72.0% growth inhibition, 50 μL of 1 mg/mL, ROS induction	Massawe et al. (2018)
520108	2-Acetyl thiazole	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of sporangial germination (min. 30% inhibition)	De Vrieze et al. (2015)
12741	2-Decanone	*Bacillus* sp.	*Fusarium solani*, 100% growth inhibition, 200 μL of pure compound	Guevara-Avendaño et al. (2019)
22556	2-Dodecanone	*Bacillus* sp.	*Fusarium solani*, 38.5% growth inhibition, 200 μL of pure compound	Guevara-Avendaño et al. (2019)

22556	2-Dodecanone	*Pseudomonas* sp.	*Fusarium solani*, 100% growth inhibition, 200 µL of pure compound	Guevara-Avendaño et al. (2019)
8051	2-Hepatanone	*Bacillus amyloliquefaciens* L3	*Fusarium oxysporum* f. sp. *niveum*; inhibition of mycelial growth; 50 µL of pure compound	Wu et al. (2019)
13187	2-Nonanone	*Bacillus amyloliquefaciens* L3	*Fusarium oxysporum* f. sp. *niveum*; inhibition of mycelial growth; 50 µL of pure compound	Wu et al. (2019)
13187	2-Nonanone	*Bacillus* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 4.9, 16.4 mg, respectively	Giorgio et al. (2015)
13187	2-Nonanone	*Bacillus* sp.	*Fusarium solani*, 100% growth inhibition, 200 µL of pure compound	Guevara-Avendaño et al. (2019)
13187	2-Nonanone	*Burkholderia ambifaria*	Growth reduction *Rhizoctonia solani*, *Alternaria alternata*, no growth reduction *Fusarium solani*, 1 ng, 1 µg, 1 mg	Groenhagen et al. (2013)
13187	2-Nonanone	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*, dry mass reduction, 100 ppm	Briard et al. (2016)
13187	2-Nonanone	*Pseudomonas chlororaphis* 449	*Rhizoctonia solani*, growth inhibition, 10–100 µmol	Popova et al. (2014)
13187	2-Nonanone	*Pseudomonas* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 4.9, 16.4 mg, respectively	Giorgio et al. (2015)
8093	2-Octanone	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. (2015)
6054	2-Phenyl-ethanol	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. 2015
8163	2-Undecanone	*Bacillus amyloliquefaciens* VM42, *Bacillus velezensis* VM10, VM11, endophytes	*Sclerotinia sclerotiorum*, 98.1% growth inhibition, 50 µL of 1 mg/mL, ROS induction, MIC 0.2 µg/mL	Massawe et al. (2018)

(continued)

Table 2.2 (continued)

Pubchem_id	Compound	Emitter	Receiver_Fungi	References
8163	2-Undecanone	*Bacillus* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 14.8, 16.5 mg, respectively	Giorgio et al. (2015)
8163	2-Undecanone	*Burkholderia ambifaria*	Growth reduction *Rhizoctonia solani*, *Alternaria alternata*, no growth reduction *Fusarium solani*, 1 ng, 1 µg, 1 mg	Groenhagen et al. (2013)
8163	2-Undecanone	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*, dry mass reduction, 100 ppm	Briard et al. (2016)
8163	2-Undecanone	*Pseudomonas chlororaphis* 449	*Rhizoctonia solani*, growth inhibition, 10–100 µmol	Popova et al. (2014)
8163	2-Undecanone	*Pseudomonas* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 14.8, 16.5 mg, respectively	Giorgio et al. (2015)
8163	2-Undecanone	*Xanthomonas campestris* pv. *vesicatoria* 85-10	Mycelium growth promotion *Rhizoctonia solani*, 0.01–100 µmol	Weise et al. (2012)
26808	2,3,5-Trimethyl pyrazine	*Bacillus* sp.	*Fusarium solani*, 100% growth inhibition, 200 microl of pure compound	Guevara-Avendaño et al. (2019)
11491990	2,5-Bis (1-methylethyl) pyrazine	*Burkholderia* sp.AD24 + *Paenibacillus* sp. AD87	*Candia albicans* BSMY212; growth inhibition, 1.84 mg	Janssens et al. (2019)
11491990	2,5-Bis (1-methylethyl) pyrazine	*Burkholderia* sp.AD24 + *Paenibacillus* sp. AD87	*Fusarium culmorum* PV; growth inhibition, 1.84 mg	Janssens et al. (2019)
11491990	2,5-Bis (1-methylethyl) pyrazine	*Burkholderia* sp.AD24 + *Paenibacillus* sp. AD87	*Rhizoctonia solani* AG2.2IIIB; growth inhibition, 1.84 mg	Janssens et al. (2019)

31252	2,5-Dimethyl pyrazine	*Bacillus pumilus* (S32)	*Phaeomoniella chlamydospora* antifungal activity, 100–2000 mg/L	Haidar et al. (2016)
31252	2,5-Dimethyl pyrazine	*Paenibacillus* sp (S19)	*Phaeomoniella chlamydospora* antifungal activity, 100–2000 mg /L	Haidar et al. (2016)
31252	2,5-Dimethyl pyrazine	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*, dry mass reduction, 100 ppm	Briard et al. (2016)
11509	3-Hexanone	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of sporangia germination (min. 30% inhibition)	De Vrieze et al. (2015)
11516	4-Octanone	*Burkholderia ambifaria*	Growth reduction *Rhizoctonia solani*, no growth reduction *Fusarium solani*, 1 ng, 1 μg, 1 mg	Groenhagen et al. (2013)
11516	4-Octanone	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. (2015)
178	Acetamide	*Alcaligenaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 2–4 g/L; mycelium growth inhibition, 53–67 g/L	Zou et al. (2007)
178	Acetamide	*Bacillales*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 2–4 g/L; mycelium growth inhibition, 53–67 g/L	Zou et al. (2007)
178	Acetamide	*Micrococcaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 2–4 g/L; mycelium growth inhibition, 53–67 g/L	Zou et al. (2007)
178	Acetamide	*Rhizobiaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 2–4 g/L; mycelium growth inhibition, 53–67 g/L	Zou et al. (2007)
178	Acetamide	*Xanthomonadaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 2–4 g/L; mycelium growth inhibition, 53–67 g/L	Zou et al. (2007)
176	Acetic acid	*Bacillus* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 4.2, 9.2 mg, respectively	Giorgio et al. (2015)

(continued)

Table 2.2 (continued)

Pubchem_id	Compound	Emitter	Receiver_Fungi	References
176	Acetic acid	*Pseudomonas* pp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 4.2, 9.2 mg, respectively	Giorgio et al. (2015)
7410	Acetophenone	*Burkholderia ambifaria*	Growth reduction of *Rhizoctonia solani*, no growth *reduction Fusarium solani*, 1 ng, 1 µg, 1 mg	Groenhagen et al. (2013)
7410	Acetophenone	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of sporangia germination (min. 30% inhibition)	De Vrieze et al. (2015)
6654	Alpha-pinene	*Bacillus amyloliquefaciens* VM42, *Bacillus velezensis* VM10, VM11, endophytes	*Sclerotinia sclerotiorum*, 34.6% growth inhibition, 50 µL of 1 mg/mL	Massawe et al. (2018)
240	Benzaldehyde	*Alcaligenaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 4–6 g/L; mycelium growth inhibition, 16–40 g/L	Zou et al. (2007)
240	Benzaldehyde	*Bacillales*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 4–6 g/L; mycelium growth inhibition, 16–40 g/L	Zou et al. (2007)
240	Benzaldehyde	*Micrococcaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 4–6 g/L; mycelium growth inhibition, 16–40 g/L	Zou et al. (2007)
240	Benzaldehyde	*Rhizobiaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 4–6 g/L; mycelium growth inhibition, 16–40 g/L	Zou et al. (2007)
240	Benzaldehyde	*Xanthomonadaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 4–6 g/L; mycelium growth inhibition, 16–40 g/L	Zou et al. (2007)
7222	Benzothiazole	*Alcaligenaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, mycelium growth inhibition, 29–158 g/L	Zou et al. (2007)
7222	Benzothiazole	*Bacillales*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, mycelium growth inhibition, 29–158 g/L	Zou et al. (2007)

7222	Benzothiazole	*Bacillus amyloliquefaciens* VM42, *Bacillus velezensis* VM10, VM11, endophytes	*Sclerotinia sclerotiorum*, 73.8% growth inhibition, 50 µL of 1 mg/mL, ROS induction, MIC 0.2 µg/mL	Massawe et al. (2018)
7222	Benzothiazole	*Micrococcaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, mycelium growth inhibition, 29–158 g/L	Zou et al. (2007)
7222	Benzothiazole	*Rhizobiaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, mycelium growth inhibition, 29–158 g/L	Zou et al. (2007)
7222	Benzothiazole	Several *Pseudomonas* sp.	*Sclerotinia sclerotiorum* mycelium growth, sclerotial germination	Fernando et al. (2005)
7222	Benzothiazole	*Xanthomonadaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, mycelium growth inhibition, 29–158 g/L	Zou et al. (2007)
7966	Cyclohexanol	Several *Pseudomonas* sp.	*Sclerotinia sclerotiorum* mycelium growth, sclerotial germination	Fernando et al. (2005)
12741	Decan-2-one	*Xanthomonas campestris* pv. *vesicatoria* 85-10	Mycelium growth inhibition *Rhizoctonia solani*, 0.01–100 µmol	Weise et al. (2012)
12232	Dimethyl disulfide	*Bacillus* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 31.4, 73.2 mg, respectively	Giorgio et al. (2015)
12232	Dimethyl disulfide	*Bacillus* sp.	*Fusarium solani*, 100 % growth inhibition, 200 µL of pure compound	Guevara-Avendaño et al. (2019)
12232	Dimethyl disulfide	*Burkholderia ambifaria*	Growth reduction *Rhizoctonia solani*, no growth reduction *Fusarium solani*, 1 ng, 1 µg, 1 mg	Groenhagen et al. (2013)
12232	Dimethyl disulfide	*Pseudomonas* sp.	*Fusarium solani*, 100% growth inhibition, 200 µL of pure compound	Guevara-Avendaño et al. (2019)
12232	Dimethyl disulfide	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*, dry mass reduction 100 ppm	Briard et al. (2016)
12232	Dimethyl disulfide	*Pseudomonas aeruginosa*	*Aspergillus fumigatus*, dry mass reduction,100 ppm	Briard et al. (2016)

(continued)

Table 2.2 (continued)

Pubchem_id	Compound	Emitter	Receiver_Fungi	References
12232	Dimethyl disulfide	*Pseudomonas* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 31.4, 73.2 mg, respectively	Giorgio et al. (2015)
12232	Dimethyl disulfide	*Pseudomonas tolaasii*	*Agaricus bisporus* discoloration, 0.156–20 µg; *Pleurotus ostreatus* mycelium growth inhibition, 1.25–20 µg	Lo Cantore et al. (2015)
19310	Dimethyl trisulfide	*Bacillus* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 24 mg	Giorgio et al. (2015)
19310	Dimethyl trisulfide	*Burkholderia ambifaria*	Growth reduction *Rhizoctonia solani*, *Alternaria alternata*; no growth *reduction Fusarium solani*, 1 ng, 1 µg, 1 mg	Groenhagen et al. (2013)
19310	Dimethyl trisulfide	*Pseudomonas* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 24 mg	Giorgio et al. (2015)
19310	Dimethyl trisulfide	Several *Pseudomonas* spp.	*Sclerotinia sclerotiorum* mycelium growth, sclerotial germination	Fernando et al. (2005)
19310	Dimethyl trisulfide	*Bacillus* sp.	*Fusarium solani*, 100% growth inhibition, 200 µL of pure compound	Guevara-Avendaño et al. (2019)
19310	Dimethyl trisulfide	*Pseudomonas* sp.	*Fusarium solani*, 100% growth inhibition, 200 µL of pure compound	Guevara-Avendaño et al. (2019)
8182	Dodecane	*Bacillus amyloliquefaciens* VM42, *Bacillus velezensis* VM10, VM11, endophytes	*Sclerotinia sclerotiorum*, 6.4% growth inhibition, 50 µL of 1 mg/mL	Massawe et al. (2018)
7361	Furfuryl alcohol	Pseudomonas sp.	*Phytophthora infestans*, inhibition of sporangia germination (min. 30% inhibition)	De Vrieze et al. (2015)
10430	Isovaleric acid	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of sporangia germination (min. 30% inhibition)	De Vrieze et al. (2015)

22311	Limonene	*Bacillus* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 17.2, 30.1 mg, respectively	Giorgio et al. (2015)
22311	Limonene	*Pseudomonas* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 17.2, 30.1 mg, respectively	Giorgio et al. (2015)
10812	m-cymene	*Bacillus* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 13.8, 17.2 mg, respectively	Giorgio et al. (2015)
10812	m-cymene	*Pseudomonas* sp.	*Sclerotinia sclerotiorum*, inhibition of mycelium growth and sclerotia formation; mic 13.8, 17.2 mg, respectively	Giorgio et al. (2015)
6329	Methanamine	*Alcaligenaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 7–9 g/L; mycelium growth inhibition, 21–33 g/L	Zou et al. (2007)
6329	Methanamine	*Bacillales*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 7–9 g/L; mycelium growth inhibition, 21–33 g/L	Zou et al. (2007)
6329	Methanamine	*Micrococcaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 7–9 g/L; mycelium growth inhibition, 21–33 g/L	Zou et al. (2007)
6329	Methanamine	*Rhizobiaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 7–9 g/L; mycelium growth inhibition, 21–33 g/L	Zou et al. (2007)
6329	Methanamine	*Xanthomonadaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 7–9 g/L; mycelium growth inhibition, 21–33 g/L	Zou et al. (2007)
878	Methanethiol	*Pseudomonas tolaasii*	*Agaricus bisporus* discoloration, 100 μg; *Pleurotus ostreatus* mycelium growth inhibition, 50 μg	Lo Cantore et al. (2015)
220639	Nitropentane	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. (2015)

(continued)

Table 2.2 (continued)

Pubchem_id	Compound	Emitter	Receiver_Fungi	References
8168	*N*,*N*-dimethyl dodecyl amine	*Bacillus amyloliquefaciens* VM42, *Bacillus velezensis* VM10, VM11, endophytes	*Sclerotinia sclerotiorum*, 58.9% growth inhibition, 50 µL of 1 mg/mL, ROS induction	Massawe et al. (2018)
31289	Nonanal	Several *Pseudomonas* sp.	*Sclerotinia sclerotiorum* mycelium growth and sclerotial germination inhibited	Fernando et al. (2005)
12391	Pentadecane	*Bacillus amyloliquefaciens* VM42, *Bacillus velezensis* VM10, VM11, endophytes	*Sclerotinia sclerotiorum*, 19.6% growth inhibition, 50 µL of 1 mg/mL	Massawe et al. (2018)
998	Phenyl acetaldehyde	*Alcaligenaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6–8 g/L; mycelium growth inhibition, 17–34 g/L	Zou et al. (2007)
998	Phenyl acetaldehyde	*Bacillales*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6–8 g/L; mycelium growth inhibition, 17–34 g/L	Zou et al. (2007)
998	Phenyl acetaldehyde	*Micrococcaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6–8 g/L; mycelium growth inhibition, 17–34 g/L	Zou et al. (2007)
998	Phenyl acetaldehyde	*Rhizobiaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6–8 g/L; mycelium growth inhibition, 17–34 g/L	Zou et al. (2007)
998	Phenyl acetaldehyde	*Xanthomonadaceae*	*Pochonia chlamydosporia*, *Paecilomyces lilacinus*, spore growth inhibition 6–8 g/L; mycelium growth inhibition, 17–34 g/L	Zou et al. (2007)
7678	Phenyl acetone	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. (2015)
11363	Phenyl propanedione	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. (2015)
7148	Propiophenone	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. (2015)

73750	*S*-Methyl-butanethioate	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of sporangia germination (min. 30% inhibition)	De Vrieze et al. (2015)
3329759952	*S*-Methyl- methane-thiosulfonate	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. (2015)
3329759952	*S*-Methyl- methane-thiosulfonate	*Burkholderia ambifaria*	Growth reduction *Rhizoctonia solani*, no growth reduction *Fusarium solani*, 1 ng, 1 μg, 1 mg	Groenhagen et al. (2013)
12388	Tridecane	Bacillus amyloliquefaciens VM42, *Bacillus velezensis* VM10, VM11, endophytes	*Sclerotinia sclerotiorum*, 11.2% growth inhibition, 50 μL of 1 mg/mL	Massawe et al. (2018)
8186	Undecanal	*Pseudomonas* sp.	*Phytophthora infestans*, inhibition of mycelium growth (min. 30% inhibition)	De Vrieze et al. (2015)

An issue which makes a comparison of the present results difficult is that in the various experiments the amounts of bacVOC applications are very different, e.g., sometimes not even indicated properly or often not in the range of ecological relevance. Furthermore, the design of the experimental set-ups is very different in different labs, e.g., different kind of container sizes with different headspace volumes were used, Petri dishes were sealed or not, or plants were illuminated by different light qualities and light regimes; these differences and many more influence the outcome/results of the experiments significantly. Standardization of experimental set-ups and designs would help to evaluate and interpret the obtained results in a collective manner to gain added-value information.

Many bacteria which release the tested compounds belong to the group of γ-proteobacteria (Lemfack et al. 2020). The scope of investigations can easily be expanded to other prokaryotic clades. This would most likely increase the chances to find new bioactive compounds in the future. In more than 50% of the experiments, the model plant *Arabidopsis* was used as receiver organism, while typical crop plants like *Brassica oleracea*, *Capsicum annum*, *Citrus* sp., *Glycine max*, *Lactuca sativa*, *Medicago* sp., *Nicotiana tabacum*, and *Zea mays* were only investigated a few times. Considering that bacVOCs may be bioactive, crop plants should be more frequently studied in the future. At a first glance, experiments of bacVOCs affecting the desert plants like *Agave* sp. are of solely scientific value, since the world population is facing a water problem in the future; however, these investigations may give some new insights by strengthening the plants' health and viability. In summary, investigations of bacVOCs affecting plants (with the hope to identify bioactive compounds) are most promising; aspects and details that further address and support this notion are found in this book, e.g., chapters written by Bailly 2020, Bruisson et al. 2020.

2.3 Bioactive Bacterial VOCs Affecting Fungi

Fungi are receivers as well as emitters of mVOCs (summarized in the *mVOC database*, Lemfack et al. 2018) and are consequently involved in VOC-mediated interactions with other organisms. Fungi often colonize or associate with plants, some are phytopathogenic. They also grow on and in animals (including humans) and some of them may cause diseases in those hosts. These pathogenic fungi have been in the focus regarding VOC research, because there is justified claim that bacVOCs may act as biological control agents (BCAs) on pathogenic fungi; e.g., *Aspergillus flavus*, *Candida albicans*, *Fusarium solani*, *Fusarium oxysporum*, *Pochonia chlamydosporia*, *Paecilomyces lilacinus*, *Phytophthora infestans*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* were preferentially studied (Table 2.2). Reduction of mycelium growth or spore germination was reported most frequently, indicating that indeed bacterial VOCs can act in a fungistatic or fungicidal manner, and subsequently may improve or promote plant or animal growth indirectly. The observations have to be handled with care since some publications do not deliver

information regarding the amount of applied bacVOCs. The observations have to be handled with care since some publications do not deliver information regarding the amount of applied bacVOCs and additionally the tested amounts may not reflect biological relevant concentrations. Furthermore experimental set-ups used for the analysis were not comparable (further arguments see previous paragraph 'Bioactive bacterial VOCs affecting plants').

In sum, 122 separate applications with 50 bacVOCs were examined so far (Table 2.2). The most frequent studied bacVOCs are 1-butane amine, 1-decene, 2-nonanone, 2-undecanone, acetamide, benzaldehyde, benzothiazole, dimethyl disulfide, dimethyl trisulfide, methanamine, and phenyl acetaldehyde, which all represent reactive organic compounds (ketones, aldehydes, amines, and sulfur compounds). Details are summarized in Table 2.2. The so far manageable number of bioactive bacVOCs justifies enlargement of bacVOC tests; systematic and comparable investigations of, e.g., LD_{50} values are favored to highlight valuable BCAs.

Up until now, knowledge about changes at the molecular or biochemical level in fungi that were fumigated by discrete bacVOCs. It would be of great interest to observe which alterations at the level of gene expression or physiology result in reduction of growth or spore germination, or are responsible for discolorations. Exploring and examining the functional modes of the bacVOCs in the receiving fungi is a prerequisite for proper medical and agricultural applications.

2.4 Bioactive Bacterial VOCs Affecting Bacteria

Many bacteria live in prokaryotic communities in nature. The microbiome research in the past clearly demonstrated how complex these microbial communities are. Presently, we are far away from understanding the population dynamics within these microbiomes, but it is very likely that the presence of molecules/metabolites influences inter- as well as intraspecific interactions. Secondary/specialized metabolites are known to be produced by bacteria to compete with other bacteria living in the same ecological niche using the same space and nutrients (Gray et al. 2019). Recently it became clear that mVOCs play an important role in structuring the environment of the communities (Kai and Piechulla 2018; Kai et al. 2018). Nevertheless, the present knowledge of bioactive bacVOCs influencing bacteria is limited since so far only 21 compounds have been investigated (Table 2.3). From the wealth of known bacteria, bioactive VOCs of species of only 11 genera were tested: *Bacillus* sp., *Bacteroides* sp., *Burkholderia* sp., *Collimonas* sp., *Escherichia coli*, *Paenibacillus* sp., *Pseudomonas* sp., *Serratia* sp., *Staphylococcus* sp., *Streptomyces* sp., *Veillonella* sp. Similarly, the number of known receiver bacteria is also quite low, comprising *Agrobacterium tumefaciens*, *Bacillus* sp., *Burkholderia* sp., *Clostridium* sp., *E. coli*, *Micrococcus* sp., *Pseudomonas* sp., *Salmonella* sp., *Synechococcus* sp., *Vibrio fischeri*, and *Xanthomonas* sp. Many more bacteria should be investigated in the future, taking into account that 10^{16} bacterial species are expected to exist on earth.

Table 2.3 Bacterial VOCs affecting bacteria

Pubchem_id	Compound	Emitter	Receiver_Bacteria	References
263	1-Butanol	Bacteria	*Escherichia coli*, *Pseudomonas aeruginosa* motility influenced	Létoffé et al. (2014)
11952	2-Aminoacetophenone	*Burkholderia ambifaria*	*Escherichia coli* resistance to amp, km, Gm; 1 ng, 1 µg, 1 mg	Groenhagen et al. (2013)
11952	2-Aminoacetophenone	*Pseudomonas aeruginosa*	Enhancing antibiotic tolerance; altering bacterial translation	Que et al. (2013), Cox and Parker (1979)
11952	2-Aminoacetophenone	*Pseudomonas aeruginosa*	*Vibrio fischeri*, lux R of the quorum sensing system is affected	Kviatkovski et al. (2015)
6569	2-Butanone	Bacteria	*Escherichia coli*, *Pseudomonas aeruginosa* motility influenced	Létoffé et al. (2014)
8051	2-Heptanone	*Pseudomonas chlororaphis* 449	*Agrobacterium tumefaciens*, inhibition of biofilm formation, 100 µmol	Plyuta et al. (2016)
8051	2-Heptanone	*Pseudomonas fluorescens* B-4117	*Agrobacterium tumefaciens*, inhibition of biofilm formation, 100 µmol	Plyuta et al. (2016)
13187	2-Nonanone	Not specified	*Synechococcus* sp. PCC7942, dissociation of antenna from PSII reaction center, disturbed regulation of state2 to state1 transition, inhibition of electron transfer from QA to QB, inhibition of electron transport at donor side of PSII, trigger detachment of phycobilisomes from photosynthetic membrane, chlorophyll degradation, 0.2–5 mM	Voronova et al. (2019)
13187	2-Nonanone	*Pseudomonas fluorescens* B-4117	Agrobacterium tumefaciens, inhibition of biofilm formation, 5–35 µmol	Plyuta et al. (2016)
8163	2-Undecanone	Not specified	*Synechococcus* sp. PCC7942, dissociation of antenna from PSII reaction center, disturbed regulation of state2 to state1 transition, inhibition of electron transfer from QA to QB, inhibition of electron transport at donor side of PSII, trigger detachment of phycobilisomes from photosynthetic membrane, chlorophyll degradation, 0.02–0.5 mM	Voronova et al. (2019)

8163	2-Undecanone	*Pseudomonas fluorescens* B-411	*Agrobacterium tumefaciens*, inhibition biofilm formation, 25–600 μmol	Plyuta et al. (2016)
650	2,3 Butanedione	*Bacillus subtilis*	*Burkholderia glumae*, *Pseudomonas aeruginosa*, *Paenibacillus polymyxa* reduction of surface motility; *Escherichia coli* motility gene expression inhibited; antibiotic resistance altered in *E. coli*, 10 nM to 1 μM	Kim et al. (2013)
262	2,3-Butanediol	*Serratia marcescens*	*Pseudomonas aeruginosa*, enhanced virulence	Venkataraman et al. (2014)
11491990	2,5-Bis(1-methylethyl) pyrazine	*Burkholderia* sp. AD24 + *Paenibacillus* sp. AD87	*Escherichia coli* WA321; growth inhibition, 1.84 mg	Janssens et al. (2019)
11491990	2,5-Bis(1-methylethyl) pyrazine	*Burkholderia* sp. AD24 + *Paenibacillus* sp. AD87	*Staphylococcus aureus* 533R4; growth inhibition, 1.84 mg	Janssens et al. (2019)
18938	3,5,5-Trimethyl hexanol	*Bacillus* sp. D13	*Xanthomonas oryzae* pv. *oryzae* growth inhibition, 2.4–4.0 mg	Xie et al. (2016)
176	Acetic acid	Bacteria	Stimulates bacterial biofilm formation	Chen et al. (2015)
176	Acetic acid	*Bacteroides fragilis*	Reduction of heat resistant spores, prevention of spore formation of *Salmonella typhimurium*, *Salmonella enteritidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Clostridium difficile*	Hinton and Hume (1995)
176	Acetic acid	*Veillonella* sp.	Reduction of heat resistant spores, prevention of spore formation of *Salmonella typhimurium*, *Salmonella enteritidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Clostridium difficile*	Hinton and Hume (1995)
179	Acetoin	Bacteria	*E. coli*, *Pseudomonas aeruginosa*, motility influenced	Létoffé et al. (2014)
7505	Benzonitrile	*Collimonas pratensis* Ter91	*Pseudomonas fluorescens* Pf0-1 growth stimulation, >3 μmol	Garbeva et al. (2014)
7505	Benzonitrile	*Serratia plymuthica* PRI-2C	*Pseudomonas fluorescens* Pf0-1 growth stimulation, >3 μmol	Garbeva et al. (2014)

(continued)

Table 2.3 (continued)

Pubchem_id	Compound	Emitter	Receiver_Bacteria	References
8174	Decyl alcohol	*Bacillus* sp. D13	*Xanthomonas oryzae* pv. *oryzae* growth inhibition, 0.48–0.8 mg	Xie et al. (2016)
12232	Dimethyl disulfide	*Collimonas pratensis* Ter91	*Pseudomonas fluorescens* Pf0-1 growth stimulation, >3 µmol	Garbeva et al. (2014)
12232	Dimethyl disulfide	*Pseudomonas fluorescens* B4117; Q8rl-96	*Agrobacterium tumefaciens*, *Agrobacterium vitis*, 0.01 to 0.1 mmol; inhibition of biofilm formation 50-100 µmol	Dandurishvili et al. (2011), Plyuta et al. (2016)
12232	Dimethyl disulfide	*Serratia plymuthica* IC1270	*Agrobacterium tumefaciens*, *Agrobacterium vitis*, 0.01–0.1 mmol; inhibition of biofilm formation 50–100 µmol	Dandurishvili et al. (2011), Plyuta et al. (2016)
12232	Dimethyl disulfide	*Serratia plymuthica* IC1270	*Agrobacterium tumefaciens*, inhibition of biofilm formation; 50–100 µmol	Plyuta et al. (2016)
12232	Dimethyl disulfide	*Serratia plymuthica* PRI-2C	*Pseudomonas fluorescens* Pf0-1 growth stimulation, >3 µmol	Garbeva et al. (2014)
760	Glyoxylic acid	*Bacillus subtilis*	*Burkholderia glumae*, *Pseudomonas aeruginosa*, *Paenibacillus polymyxa* reduction of surface motility; *Bacillus*, *Escherichia coli* motility gene expression inhibited; altered antibiotic-resistance in *E. coli*	Kim et al. (2013)
798	Indole	Bacteria	*Escherichia coli*, *Pseudomonas aeruginosa* motility influenced	Létoffé et al. (2014)
798	Indole	*Escherichia coli*	Interbacterial communication, inhibits biofilm formation	DiMartino et al. (2003)
798	Indole	*Escherichia coli*	*Pseudomonas putida*, induction of efflux pump	Molina-Santiago et al. (2014)
798	Indole	Many gram-positive + gram-negative bacteria	Spore formation, signaling, plasmid stability, drug resistance, biofilm formation virulence in *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enterica*	Lee and Lee (2010)

6590	Isobutyrate	*Bacteroides fragilis*	Inhibition of growth and sporulation *Clostridium perfringens*, 100 mM	Wrigley (2004)
263	*n*-Butanol	*Pseudomonas taiwanensis* VLB120C	*Pseudomonas taiwanensis* VLB120C, inhibition of biofilm formation, 3%	Halan et al. (2016)
1032	Propionic acid	*Bacteroides fragilis*	Reduction of heat resistant spores, prevention of spore formation of *Salmonella typhimurium*, *Salmonella enteritidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Clostridium difficile*	Hinton and Hume (1995)
1032	Propionic acid	*Veillonella* sp.	Reduction of heat resistant spores, prevention of spore formation of *Salmonella typhimurium*, *Salmonella enteritidis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Clostridium perfringens*, *Clostridium difficile*	Hinton and Hume (1995)
137333887	Schleiferon A (3-(phenylamino)-butan-2-one	*Staphylococcus schleiferi*	G+ : Growth inhibition, G− : Prodigiosin and bioluminescence inhibition	Lemfack et al. (2016)
-	Schleiferon B 3-(phenylimino)-butan-2-one)	*Staphylococcus schleiferi*	G+ : Growth inhibition, G− : Prodigiosin and bioluminescence inhibition	Lemfack et al. (2016)
1146	Trimethyl amine	*Bacillus subtilis*	Antibiotic resistance/tolerance of G+, G− increased nonspecific transient alteration of antibiotic uptake due to pH increase in environment, affecting community behavior	Létoffé et al. (2014)
1146	Trimethyl amine	*Escherichia coli*	Antibiotic resistance/tolerance of G+, G− increased nonspecific transient alteration of antibiotic uptake due to pH increase in environment, affecting community behavior	Létoffé et al. (2014)
1146	Trimethyl amine	*Pseudomonas aeruginosa*	Antibiotic resistance/tolerance of G+, G− increased nonspecific transient alteration of antibiotic uptake due to pH increase in environment, affecting community behavior	Létoffé et al. (2014)

(continued)

Table 2.3 (continued)

Pubchem_id	Compound	Emitter	Receiver_Bacteria	References
1146	Trimethyl amine	*Staphylococcus aureus*	Antibiotic resistance/tolerance of G+, G− increased nonspecific transient alteration of antibiotic uptake due to pH increase in environment, affecting community behavior	Létoffé et al. (2014)
1146	Trimethyl amine	*Streptomyces venezuelae*	Growth inhibition of *Micrococcus luteus* and *Bacillus subtilis*, 0.9–22.5% TMA, alkalization of the medium, induced morphological alterations, developmental switch, induction of explorer growth	Jones et al. (2017)

Table 2.3 summarizes the so far recorded influences of bacVOCs on bacteria. Motility alterations as well as growth stimulation and inhibition of bacteria were observed, however, the responses seemed to be rather unspecific. The bacVOC ketones 2-nonanone and 2-undecanone alter the electron transfer within the PSII reaction center and subsequently affect photosynthetic activity in cyanobacteria. Interest focusses on experiments with human and plant pathogenic bacteria, both as emitters and receivers. The search for bacVOC signatures which can be used as biomarkers or fingerprints of emitters would be helpful in establishing fast and non-invasive diagnostic tools. Indeed ketones (2-aminoacetophenone, 2-undecanone, and others), sulfur, and nitrogen containing compounds (dimethyl disulfide and trimethylamine, respectively) inhibit biofilm formation and influence antibiotic resistance, which are both pathogenicity-related phenomena.

It is a challenge for the future to perform meaningful experiments with more than one pure bacterial culture. VOC analysis of the headspace of co-cultures is feasible in, e.g., microtiter plates or VOC collection systems (Kai et al. 2018). The number of co-cultivated bacteria can be gradually increased as well as the combination of bacteria. However, it can be foreseen that a species-complexity as it is present in the nature can never be reached in lab experiments. Nevertheless, even in experiments where bacteria do not intermingle as in nature, interesting questions can be addressed and principles be proven like: Do bacVOCs alter the VOC profile of other bacteria qualitatively and quantitatively during co-cultivation? Do new compounds appear? Since the VOC profiles contain reactive compounds such as ketones, it is also possible that chemical reactions appear in the headspace of culture flasks or Petri dishes that are used in the lab, or in natural cavities in the habitat. This principle was recently demonstrated by the interspecific formation of the antimicrobial volatile schleiferon (Kai et al. 2018). Schleiferon A and B are structurally novel ketones (3-(phenylamino)butan-2-one and 3-(phenylimino)butan-2-one) released from *Staphylococcus schleiferi*. These compounds were thoroughly investigated (Lemfack et al. 2016) exhibiting growth inhibitions of Gram-positive bacteria with a LD_{50} in the range of 10–100 μg/ml. Furthermore, quorum sensing controlled phenotypes such as prodigiosin accumulation and bioluminescence of Gram-negative bacteria were altered.

2.5 Bioactive Bacterial VOCs Affecting Invertebrates

The soil is a highly populated habitat where many different organisms live and interact with each other (Wenke et al. 2010). In the past decade, belowground VOC-mediated interactions were investigated in more detail, particularly interactions with plants were addressed (Schenkel et al. 2019), while interactions with invertebrates, protozoa, etc. are less well studied (Kai et al. 2009; Schulz-Bohm et al. 2017). Experiments with discrete bacVOCs picturing the soil bacterial-invertebrate and protist interactions are still very rare.

The crop harvest worldwide is significantly (ca. 10%) reduced by plant-parasitic nematodes, e.g., *Meloidogyne* species parasitizes more than 2000 plant species (summarized in Terra et al. 2018). Commercial nematicides based on chemical products are the foremost tools to control such nematodes; however, they are extremely harmful to the environment. New eco-friendly molecules with nematicidal activity for commercial purposes are demanded. Bacterial VOCs might be such an alternative. Table 2.4 summarizes the bioactive bacVOCs that were tested on *Caenorhabditis elegans*, *Meloidogyne incognita*, *Panagrellus redividus*, and *Bursaphelenchus xylophilus*. The bioactive compounds are alcohols, aldehydes, ketones, acids, and dimethyl disulfide. bacVOCs with nematicidal activity reveal reduction of movement, inhibit development and viability, or result in death. A few compounds were also found to act on *Drosophila melanogaster*, cockroaches, *Aedes aegypti,* or *Bactrocera oleae*. Although, there is only a handful of publications describing the search and examination of bioactive bacVOCs affecting invertebrates (Table 2.4), much more indirect evidence exists, e.g., mosquitos and flies are attracted by decaying vegetables and fruits in which, due to the growth of microorganisms, carbon sources (e.g., glucose, fructose, sucrose) are metabolized to produce attractive VOCs. The resulting alcohols, aldehydes, ketones, and acids are key compounds for insects to localize sites for oviposition (Leroy et al. 2011; Ponnusamy et al. 2008).

2.6 Summary and Perspectives

In the past, it was demonstrated that small bacterial volatile metabolites influence the phenotypes of plants, animals, and microorganisms. In co-cultures, the receiver is exposed to a dynamic and complex mixture of organic and inorganic volatiles. These co-cultivations resemble a reductionistic view of the conditions present in the natural habitat. Clearly, besides the performance of dual cultivations, multi-species cultivation systems have to be investigated in the future to establish a more holistic understanding of the volatile-mediated interactions in a given ecological niche. It is a challenge to simulate the natural conditions to the best of our knowledge and still be able to analyze the variable parameters that influence a system.

Furthermore, the detection and identification of individual bioactive and specific volatile compounds is very important. Many experiments indicate that discrete compounds influence the receiving organism, but when the same compound targets different organisms the reactions are considered unspecific and are not good candidate compounds for applications because they harbor the risk that beside the target organism other species are also negatively affected. Interestingly, out of the ca. 120 discrete VOCs (Fig. 2.2), we found 29, 31, 12, 22 compounds that affect *either* plants, fungi, bacteria, or invertebrates, respectively, or were so far only tested in the respective interorganismal interaction, they are tentatively considered specific. These compounds are candidate compounds worthwhile to be tested in potential applications. The challenges for the future are the elucidation of specific molecular

Table 2.4 Bacterial VOCs affecting invertebrates

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
289151	(+)-Longifolene	Actinobacteria	Attracting nematodes	Hao et al. (2011)
289151	(+)-Longifolene	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
289151	(+)-Longifolene	Bacilli	Attracting nematodes	Hao et al. (2011)
289151	(+)-Longifolene	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
7731	1-Methoxy-4-methyl-benzene	*Achromobacter xylosoxidans* AF411019	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.075–0.3 mmol	Xu et al. (2015)
7731	1-Methoxy-4-methyl-benzene	*Proteus hauseri* JN092591	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.075–0.3 mmol	Xu et al. (2015)
7731	1-Methoxy-4-methyl-benzene	*Wautersiella falsenii* AM238687	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.075–0.3 mmol	Xu et al. (2015)
13190	1-Undecene	*Pseudomonas chlororaphis* 449	*Caenorhabditis elegans*, inhibition of viability and development, 25 μmol	Popova et al. (2014)
8051	2-Heptanone	Actinobacteria	Attracting nematodes	Hao et al. (2011)
8051	2-Heptanone	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
8051	2-Heptanone	Bacilli	Attracting nematodes	Hao et al. (2011)
8051	2-Heptanone	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
13187	2-Nonanone	*Bacillus simplex*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
13187	2-Nonanone	*Bacillus subtilis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
13187	2-Nonanone	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
13187	2-Nonanone	*Microbacterium oxydans*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
13187	2-Nonanone	*Pseudomonas chlororaphis* 449	*Caenorhabditis elegans*, inhibition of viability and development, 25 µmol; *Drosophila melanogaster* viability inhibited, 5–10 µmol	Popova et al. (2014)
13187	2-Nonanone	*Serratia marcescens*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
13187	2-Nonanone	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
13187	2-Nonanone	*Streptomyces lateritius*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
20083	2-Octanol	*Bacillus simplex*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
20083	2-Octanol	*Bacillus subtilis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
20083	2-Octanol	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
20083	2-Octanol	*Microbacterium oxydans*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
20083	2-Octanol	*Serratia marcescens*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
20083	2-Octanol	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
20083	2-Octanol	*Streptomyces lateritius*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8163	2-Undecanone	*Bacillus simplex*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8163	2-Undecanone	*Bacillus subtilis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. 2007)
8163	2-Undecanone	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8163	2-Undecanone	*Microbacterium oxydans*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8163	2-Undecanone	*Pseudomonas chlororaphis* 449	*Caenorhabditis elegans*, inhibition of viability and development, 25 µmol	Popova et al. (2014)
8163	2-Undecanone	*Serratia marcescens*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
8163	2-Undecanone	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8163	2-Undecanone	*Streptomyces lateritius*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
650	2,3 Butanedione (diacetyl)	*Bacillus subtilis*	Odr10 receptor of *Caenorhabditis elegans* specific interaction	Sengupta et al. (1996)
262	2,3-Butanediol	Bacteria	Increasing the time until female cockroaches reach parturition	Moore and Moore (2001)
7410	Acetophenone	*Achromobacter xylosoxidans* AF411019	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.086–0.344 mmol	Xu et al. (2015)
7410	Acetophenone	*Arthrobacter nicotianae* JQ071518	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.086–0.344 mmol	Xu et al. (2015)
7410	Acetophenone	*Bacillus simplex*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
7410	Acetophenone	*Bacillus subtilis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
7410	Acetophenone	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
7410	Acetophenone	*Microbacterium oxydans*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
7410	Acetophenone	*Pseudochrobacterium saccharolyticum* AM180484	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.086–0.344 mmol	Xu et al. (2015)
7410	Acetophenone	*Serratia marcescens*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
7410	Acetophenone	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
7410	Acetophenone	*Streptomyces lateritius*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
240	Benzaldehyde	Actinobacteria	Attracting nematodes	Hao et al. (2011)
240	Benzaldehyde	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
240	Benzaldehyde	Bacilli	Attracting nematodes	Hao et al. (2011)
240	Benzaldehyde	*Bacillus simplex*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
240	Benzaldehyde	*Bacillus subtilis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
240	Benzaldehyde	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrellus redividus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
240	Benzaldehyde	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
240	Benzaldehyde	*Microbacterium oxydans*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
240	Benzaldehyde	*Serratia marcescens*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
240	Benzaldehyde	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
240	Benzaldehyde	*Streptomyces lateritius*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
244	Benzyl alcohol	Actinobacteria	Attracting nematodes	Hao et al. (2011)
244	Benzyl alcohol	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
244	Benzyl alcohol	Bacilli	Attracting nematodes	Hao et al. (2011)
244	Benzyl alcohol	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
2345	Benzyl benzoate	Actinobacteria	Attracting nematodes	Hao et al. (2011)
2345	Benzyl benzoate	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
2345	Benzyl benzoate	Bacilli	Attracting nematodes	Hao et al. (2011)
2345	Benzyl benzoate	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
7981	Butyl-isovalerate	*Wautersiella falsenii* AM238687	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.054–0.216 mmol	Xu et al. (2015)
8079	Cyclohexene	*Bacillus simplex*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8079	Cyclohexene	*Bacillus subtilis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
8079	Cyclohexene	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8079	Cyclohexene	*Microbacterium oxydans*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8079	Cyclohexene	*Serratia marcescens*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8079	Cyclohexene	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8079	Cyclohexene	*Streptomyces lateritius*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8175	Decanal	*Bacillus simplex*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8175	Decanal	*Bacillus subtilis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8175	Decanal	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
8175	Decanal	*Microbacterium oxydans*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8175	Decanal	*Serratia marcescens*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8175	Decanal	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8175	Decanal	*Streptomyces lateritius*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
80308	Delta-alaninol	Actinobacteria	Attracting nematodes	Hao et al. (2011)
80308	Delta-alaninol	Alphaproteobacteria Shingobacteria	Attracting nematodes	Hao et al. (2011)
80308	Delta-alaninol	Bacilli	Attracting nematodes	Hao et al. (2011)
80308	Delta-alaninol	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
12232	Dimethyl disulfide	*Achromobacter xylosoxidans* AF411019	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.113–0.452 mmol	Xu et al. (2015)
12232	Dimethyl disulfide	*Arthrobacter nicotianae* JQ071518	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.113–0.452 mmol	Xu et al. (2015)
12232	Dimethyl disulfide	*Bacillus simplex*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
12232	Dimethyl disulfide	*Bacillus subtilis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
12232	Dimethyl disulfide	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
12232	Dimethyl disulfide	*Microbacterium oxydans*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
12232	Dimethyl disulfide	*Proteus hauseri* JN092591	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.113–0.452 mmol	Xu et al. (2015)
12232	Dimethyl disulfide	*Pseudochromobacterium saccharolyticum* AM180484	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.113–0.452 mmol	Xu et al. (2015)
12232	Dimethyl disulfide	*Serratia marcescens*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
12232	Dimethyl disulfide	*Serratia proteamaculans* 94	*Caenorhabditis elegans*, inhibition of viability and development, 25 µmol; *Drosophila melanogaster* viability inhibited, 5–10 µmol	Popova et al. (2014)
12232	Dimethyl disulfide	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
12232	Dimethyl disulfide	*Streptomyces lateritius*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
12232	Dimethyl disulfide	*Wautersiella falsenii* AM238687	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.113–0.452 mmol	Xu et al. (2015)
7762	Ethyl butyrate	Actinobacteria	Attracting nematodes	Hao et al. (2011)
7762	Ethyl butyrate	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
7762	Ethyl butyrate	Bacilli	Attracting nematodes	Hao et al. (2011)
7762	Ethyl butyrate	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
12366	Ethyl palmitate	Actinobacteria	Attracting nematodes	Hao et al. (2011)
12366	Ethyl palmitate	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
12366	Ethyl palmitate	Bacilli	Attracting nematodes	Hao et al. (2011)
12366	Ethyl palmitate	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
24851644	Ethyl-3,3-dimethyl-acrylate	*Pseudochromobacterium saccharolyticum* AM180484	*Caenorhabditis elegans, Meloidogyne incognita*, nematicidal activity, 0.071–0.284 mmol	Xu et al. (2015)
29746	Geosmin	*Streptomyces* sp.	Microbe detection in *Drosophila melanogaster*	Stensmyr et al. (2012)
12127	Isovanillin	Actinobacteria	Attracting nematodes	Hao et al. (2011)
12127	Isovanillin	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
12127	Isovanillin	Bacilli	Attracting nematodes	Hao et al. (2011)
12127	Isovanillin	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
519840	Methyl thioacetate	*Pseudomonas putida*	*Bactrocera oleae* (Rossi) electroantennogram 0.01–100 µg, electropalpgram 0.01–100 µg	Liscia et al. (2013)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
13187	Nonan-2-one	*Achromobacter xylosoxidans* AF411019	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.058–0.232 mmol	Xu et al. (2015)
13187	Nonan-2-one	*Proteus hauseri* JN092591	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.058–0.232 mmol	Xu et al. (2015)
13187	Nonan-2-one	*Pseudochromobacterium saccharolyticum* AM180484	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.058–0.232 mmol	Xu et al. (2015)
13187	Nonan-2-one	*Wautersiella falsenii* AM238687	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.058–0.232 mmol	Xu et al. (2015)
8141	Nonane	*Bacillus simplex*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8141	Nonane	*Bacillus subtilis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8141	Nonane	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8141	Nonane	*Microbacterium oxydans*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8141	Nonane	*Serratia marcescens*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
8141	Nonane	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8141	Nonane	*Streptomyces lateritius*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
8158	Nonanoic acid	Plant associated bacteria	*Aedes aegypti*, induction of egg laying in females	Ponnusamy et al. (2008)
31244	*p*-Anisaldehyde	Actinobacteria	Attracting nematodes	Hao et al. (2011)
31244	*p*-Anisaldehyde	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
31244	*p*-Anisaldehyde	Bacilli	Attracting nematodes	Hao et al. (2011)
31244	*p*-Anisaldehyde	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)
996	Phenol	*Bacillus simplex*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
996	Phenol	*Bacillus subtilis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
996	Phenol	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
996	Phenol	*Microbacterium oxydans*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
996	Phenol	*Serratia marcescens*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
996	Phenol	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
996	Phenol	*Streptomyces lateritius*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
62444	*S*-Methyl-thio-butyrate	*Achromobacter xylosoxidans* AF411019	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.068–0.272 mmol	Xu et al. (2015)
	S-Methyl-thio-butyrate	*Arthrobacter nicotianae* JQ071518	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.068–0.272 mmol	Xu et al. (2015)
62444	*S*-Methyl-thio-butyrate	*Proteus hauseri* JN092591	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.068–0.272 mmol	Xu et al. (2015)
	S-Methyl-thio-butyrate	*Pseudochromobacterium saccharolyticum* AM180484	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.068–0.272 mmol	Xu et al. (2015)
62444	*S*-Methyl-thio-butyrate	*Wautersiella falsenii* AM238687	*Caenorhabditis elegans*, *Meloidogyne incognita*, nematicidal activity, 0.068–0.272 mmol	Xu et al. (2015)
17100	Terpineol	*Bacillus simplex*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)

(continued)

Table 2.4 (continued)

Pubchem_id	Compound	Emitter	Receiver_Invertebrate	References
17100	Terpineol	*Bacillus subtilis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
17100	Terpineol	*Bacillus weihenstephanensis*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
17100	Terpineol	*Microbacterium oxydans*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
17100	Terpineol	*Serratia marcescens*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
17100	Terpineol	*Stenotrophomonas maltophilia*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
17100	Terpineol	*Streptomyces lateritius*	Reduction of movement or death of *Panagrelleus redivivus* and *Bursaphelenchus xylophilus*	Gu et al. (2007)
11005	Tetradecanoic acid methyl ester	Plant associated bacteria	*Aedes aegypti*, induction of egg laying in females	Ponnusamy et al. (2008)
6271	Vanatone	Actinobacteria	Attracting nematodes	Hao et al. (2011)
6271	Vanatone	Alphaproteobacteria Sphingobacteria	Attracting nematodes	Hao et al. (2011)
6271	Vanatone	Bacilli	Attracting nematodes	Hao et al. (2011)
6271	Vanatone	Gammaproteobacteria	Attracting nematodes	Hao et al. (2011)

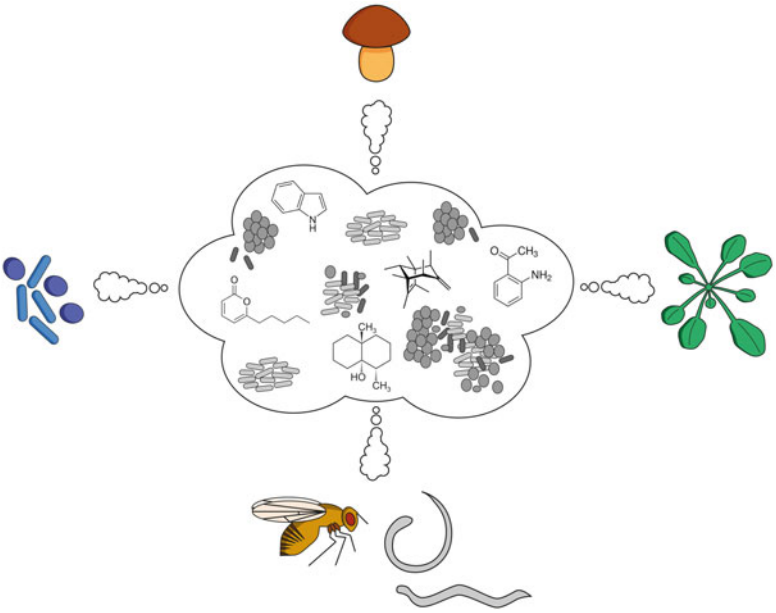

Fig. 2.1 Bacterial volatiles interact with fungi, plants, animals, and microbial communities and thereby structure and influence habitats, communities, and populations

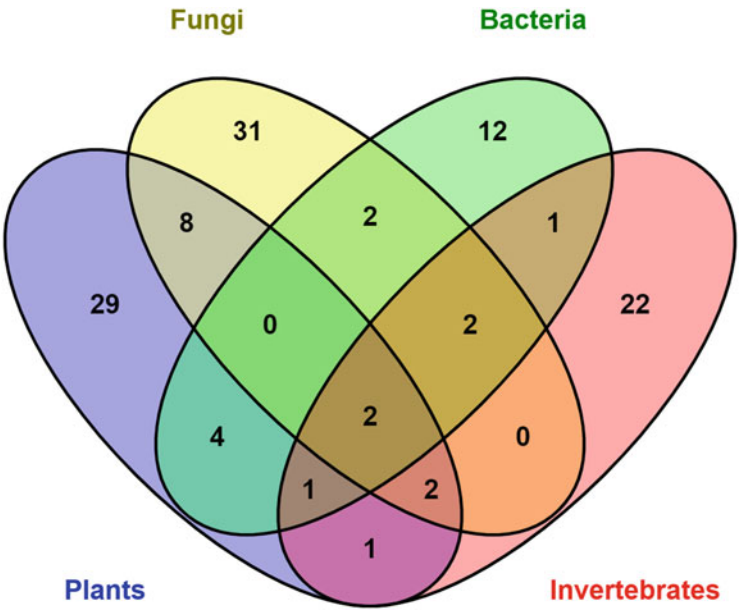

Fig. 2.2 VENN diagram showing the number of discrete VOCs in the different spaces. Eighty-four mVOCs are tentatively considered specific since so far only effects on either plants, fungi, bacteria, or invertebrate were reported. Twenty-two mVOCs affect two or more organisms

targets and the determination of bioactive doses relevant in each organismal interaction. Particularly the latter is important to understand the ecosystem and perform proper simulation and test experiments in the lab or field. Proof-of-concepts have been performed which indicate that these strategies are feasible, but more and detailed analyses are needed to reach the ultimate goals. Finally, the search for yet unknown and complex compounds applicable in agriculture and animal and human health care continuously have to be put forward.

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Chapter 3
Structural Diversity of Bacterial Volatiles

Stefan Schulz, Christian Schlawis, Diana Koteska, Tim Harig, and Peter Biwer

Abstract Bacterial volatile organic compounds (VOCs) represent a structurally very broad mixture of compounds, likely not less diverse than plant or animal volatiles. Within this diversity individual compounds might be commonly found in many, often unrelated strains, while others are restricted to a certain group of strains. In addition, strain specific compounds occur. In the following chapter we will describe the reason for this diversity, the use of different biosynthetic pathways for the production of bacterial VOCs. The use of the primary metabolism machinery, the fatty acid and polyketide biosynthetic pathways, the sulfur metabolism, pyrazine and aromatic compound formation, and terpene biosynthesis will be described and the formation of important representative compounds will be discussed.

Keywords Fatty acid derivatives · Amino acid derivatives · Terpenes · Sulfur compounds · Aromatic compounds · Lactones

3.1 Introduction

Bacterial volatiles show high structural variability leading to an enormous number of compounds reported so far (Schulz and Dickschat 2007; Lemfack et al. 2018). Before taking a tour through the chemistry and biosynthesis of these diverse structures, the term volatile compound, which are mostly organic (VOC), has to be defined. A simple definition uses the headspace above a bacterial culture as defining space. Every compound that can be trapped from the gas phase (headspace) above the culture is therefore volatile. This can be easily checked by gas chromatography/mass spectrometry (GC/MS), indicating a molecular weight limit around 300 Da. Usually only one or two functional groups are present, adding to a very often lipophilic character of the VOCs. The 300 Da limit is markedly reduced when the

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compound is polar due to strong H-bonding, as found, e.g., in amines, diols, or cyclic dipeptides (Groenhagen et al. 2014). Surprisingly, lipophilic VOCs are also emitted into the water phase. Although water solubility is usually low, small amounts of the compounds are dissolved and diffuse through the water phase. This diffusion is believed to be fast (Boland 1995) because most VOCs have a low effective size. This effect originates from their low number of functional groups that hinders hydration due to missing H-bond acceptors or multiple polarized bonds. This is in contrast to other compounds such as antibiotics that usually show a fair number of functional groups, leading to a larger hydration sphere and therefore to a larger effective size, reducing diffusion rates.

Although the structural diversity of bacterial VOCs is high, their abundance among the producing strains varies widely. Some compounds are produced randomly by many, even non-related strains, while others are restricted to few or even single strains. Therefore, one can divide bacterial VOCs into the three following classes.

- *Common compounds* occur randomly throughout the bacterial kingdom. This does not mean that they are produced by every strain, but they have often been reported from diverse bacterial groups. Nevertheless, strain or species-specific mixtures can be formed that can have biological effects, differing depending on the exact composition. Included here are mostly derivatives originating from the primary metabolism, but also sulfur compounds such as dimethyl disulfide (DMDS), aromatic compounds such as 2-phenylethanol, indole, and also aliphatic methyl ketones.
- *Group compounds* are released by certain species, genera, or families. Examples are the terpenoids geosmin and 2-methylisoborneol, typically reported from actinobacteria, myxobacteria, and cyanobacteria only. 2,5-Bis(isopropyl)-Diisopropylpyrazine also belongs to this class, because only specific strains produce this compound, even if the families are not related, e.g., *Paenibacillus* (Beck et al. 2003) and *Chondromyces* (Dickschat et al. 2007).
- *Singular or specific compounds* are produced only by one or few closely related strains. These compounds attract special interest, because their often non-unique structures imply a specific biological function. They can originate from diverse biosynthetic pathways, e.g., the polyketide streptopyridine from *Streptomyces* (Groenhagen et al. 2014), cyclohexenyl esters from the shikimate pathway of *Salinispora tropica* (Groenhagen et al. 2016), and the terpenoid sodorifen (von Reuß et al. 2010).

In the following sections we will describe VOCs originating from the diverse biosynthetic pathways found in bacteria. A general overview of all compounds will not be given because a very useful online database is available that lists compounds and producing organisms and allows extensive searches (Lemfack et al. 2018). A fundamental review describes many volatile compounds found in bacteria (Schulz and Dickschat 2007).

3.2 Derivatives of the Primary Metabolism

Almost all bacteria emitting VOCs release compounds derived directly from the primary metabolism. This includes typical fermentation products and other, usually small compounds. Important starting points are aliphatic amino acids such as alanine, valine, leucine, and isoleucine (**1**) (Fig. 3.1). Transamination of the amino acids **1** leads to keto acids **2** that lose CO_2 to yield aldehydes **4**. These aldehydes can either be oxidized to form, e.g., 2- and 3-methylbutanoic as well as isobutyric acids **6**, or they are reduced to deliver instead the respective alcohols **5** (Smit et al. 2005). Such alcohols and acids can be fused into esters usually by acyltransferases, leading to short chain esters **7**, but other pathways to esters also exist (Kruis et al. 2019). Alternatively, the nitrogen of the amino acids **1** can be retained and decarboxylation leads to primary amines **3**. Further modification of these compounds occurs often, e.g., terminal oxidation of acids such as 3-methylbutanoic acid (**6a**) and 2-methylbutanoic acid (**6b**) followed by ring closure, leading to lactones such as 3-methyl-4-butanolide (**13**) and 2-methyl-4-butanolide (**14**) (Fig. 3.2) (Citron et al. 2015).

The amines **3** can react with various compounds to form a range of *N*-volatiles. Reaction with aldehydes (**4**) or acids (**6**) leads to imines **10** and amides **9**, while other

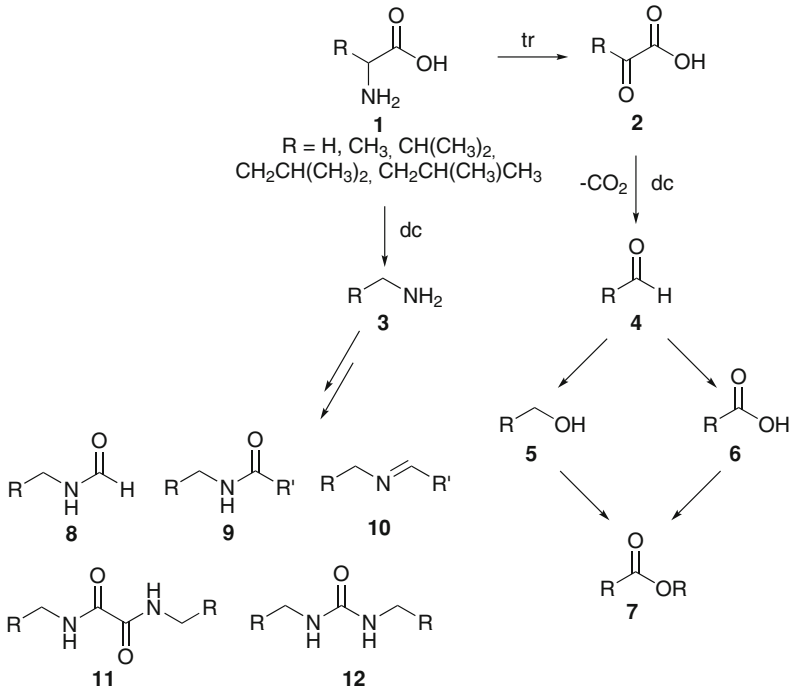

Fig. 3.1 Biosynthesis of short chain volatile compounds from amino acids. *tr* transamination, *dc* decarboxylation

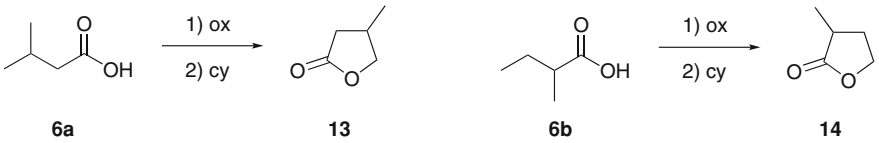

Fig. 3.2 3-Methylbutyric acid (**6a**) derived from leucine and 2-methylbutyric acid (**6b**) are the precursors of 3-methyl-4-butanolide (**13**) and 2-methyl-4-butanolide (**14**). They are formed by terminal oxidation (ox), followed by ring closure (cy)

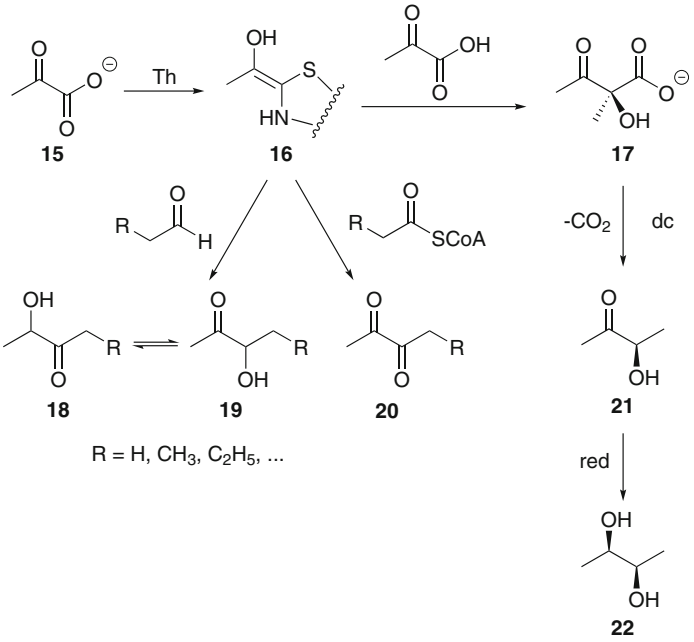

Fig. 3.3 Biosynthesis of diketones (**20**), ketols (**18**, **19**, **21**), and diols (**22**). *Th* thiamine, *dc* decarboxylation, *red* reduction

reactions furnish formamides (**8**), oxalamides (**11**), and *N*,*N*-dialkylureas (**12**) (Harig et al. 2017). One carbon chain elongation sometimes results in elongated versions of the short chain compounds discussed, as in *N*-isopentyl-4-methylpentanamide, formed by a one carbon elongation of 3-methylbutanoic acid. Finally, ammonia can be released from amino acids by various bacteria (Xiang and Moore 2005).

Other volatiles originate from the well-known fermentation of lactate, carbohydrates, and other precursors (Schomburg and Michal 2012). The resulting compounds include, e.g., ethanol, acetone, butanone, 1-butanol, acetic and butyric acids, and many other small compounds. Of special interest is the formation of acetoin (**21**), occurring in many bacteria (Fig. 3.3). Pyruvate (**15**) is activated by thiamine to form the intermediate **16** that reacts with another pyruvate molecule to form acetolactate **17**. The following decarboxylation leads to (*R*)-acetoin (**21**). Finally,

reduction furnishes (2*R*,3*R*)-butane-2,3-diol (**22**) in case of *Bacillus polymyxa* (Mas et al. 1988). Other bacteria can produce different stereoisomers. The key intermediate **16** can also react with acetyl-CoA to diacetyl (**20**). Alternatively, reaction with an aldehyde might lead to the ketol **19**. If the two substituents of **19** are different, two isomers exist that interconvert easily (Nielsen et al. 2010). By these pathways higher homologs of acetoin-like products are formed, such as 3-hydroxy-2-pentanone (Groenhagen et al. 2016), 3-hydroxy-2-hexanone and their respective isomers, 2-hydroxy-3-pentanone and 2-hydroxy-3-hexanone (Lee et al. 2012), or 2,3-hexanedione (Höckelmann et al. 2004).

These and the other small metabolites derived from the primary metabolism usually belong to the class of common compounds. They occur in many bacteria, without indicating any phylogenetic relationships. Nevertheless, the emitting strain usually releases only some of these compounds in specific amounts, thus potentially leading to a VOC profile that may be typical for this strain or genus (Choudoir et al. 2019). The VOC profile might also be unspecific in other cases.

3.3 The Fatty Acid Biosynthetic Pathway

The fatty acid biosynthetic pathway functions as large structurally variable pool of VOCs. This pathway gives access to a wide variety of structural motifs and chain lengths, potentially available for VOCs serving a specific purpose. Variations occur in the alkyl chain length, number and position of methyl branches, and oxidative status of the functional group that is usually located at or near one end of the compound.

The general, well-established biosynthetic pathway leading to bacterial fatty acids is shown in Fig. 3.4 (Schomburg and Michal 2012). Common fatty acids are produced from acetyl-CoA used as starter, followed by several cycles of chain elongation with malonyl-CoA terminating at the required chain length with a thioesterase that liberates the respective acids. Diversity of compounds arises by two important mechanisms. The first one is modification of the starter unit. Instead of acetyl-CoA, many bacteria use starters derived from the aliphatic amino acids (see above), branched 2- and 3-methylbutanoyl, isobutanoyl, or propanoyl-CoA thioesters, leading to terminally branched or one-carbon extended reduced ends of the alkyl chain. Secondly, the intermediates of every extension cycle can be decarboxylated. This transformation leads from typical acyl precursors to alkanes, 1-alkenes, and methyl ketones (Fig. 3.6). Secondary 2-alkanols are most likely not formed via decarboxylation, but by reduction of the respective methyl ketones. Because of the loss of one carbon during decarboxylation, precursors with an even number of carbons in the chain lead to odd numbered volatiles, e.g., 3-oxodecanoic acid is the precursor of 2-nonanone. Terminally functionalized compounds, such as acids, 1-alkanols, and aldehydes, can be formed from acyl-CoA precursors by hydrolysis followed by reduction or by direct reduction. Such volatiles can be theoretically formed by the fatty acid biosynthetic machinery either during

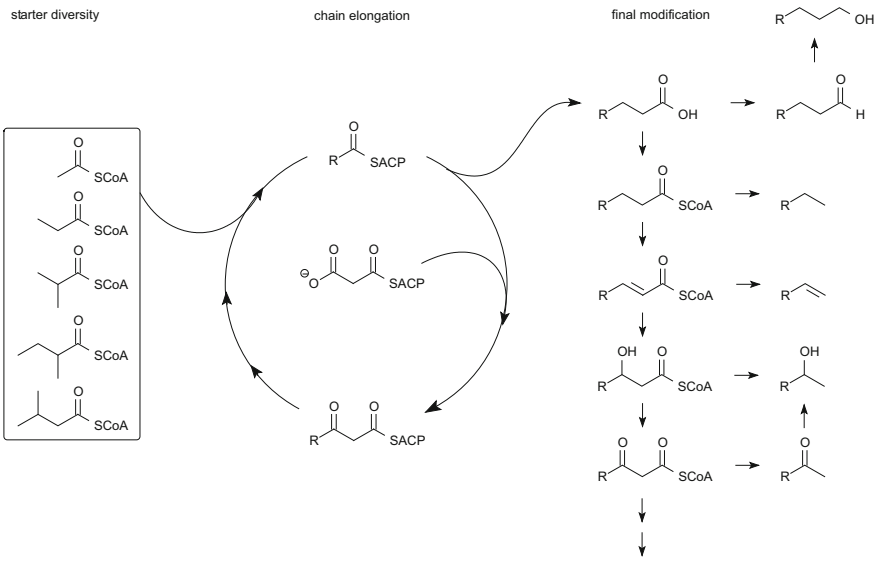

Fig. 3.4 General biosynthesis of fatty acid derivatives

anabolism or catabolism. A striking example of the latter is the formation of 1-undecene (**23**) during degradation of fatty acids by *Pseudomonas* strains (Fig. 3.5). Stable isotope labeling showed that palmitic acid is first degraded to octanoic acid, which is in turn transformed into **23**, requiring two elongation rounds with malonate and a reductive decarboxylation of a 2-dodecenoyl-CoA or free acid precursor (Montes Vidal et al. 2017).

Decarboxylation of a ketocarboxyl intermediate furnishes methyl ketones, which are frequently found in bacteria, but also the corresponding alcohols or esters are common (Dickschat et al. 2005a, 2004; Weise et al. 2012). A study on volatiles of bacteria of the *Cytophaga*-Flavobacteria-Bacteroides phylum revealed high structural diversity of methyl ketones by use of different starter compounds, chain length, and introduction of double bonds as an additional diversification process. More than 20 different C_{12}-C_{16}-ketones were identified (Dickschat et al. 2005a), including some ethyl ketones. Related secondary alcohols occur as well, presumably formed by reduction of the ketones. Similarly, the unrelated strain *Xanthomonas campestris* produces many ketones, 10-methylundecan-2-one (**24**) being the most abundant (Weise et al. 2012). The biosynthesis of an ethyl ketone, 9-methyl-3-decanone (**39**), in the myxobacterium *Myxococcus xanthus* was investigated in detail by isotope labeling (Fig. 3.6) (Dickschat et al. 2004). The starter unit 3-methylbutyrate (**36**) from leucine is elongated by two malonate units, followed by methylmalonate addition to form 2,9-dimethyl-3-oxodecanoate (**38**). Finally, decarboxylation releases ethyl ketone **39**. α-Oxidation of fatty acids can also lead

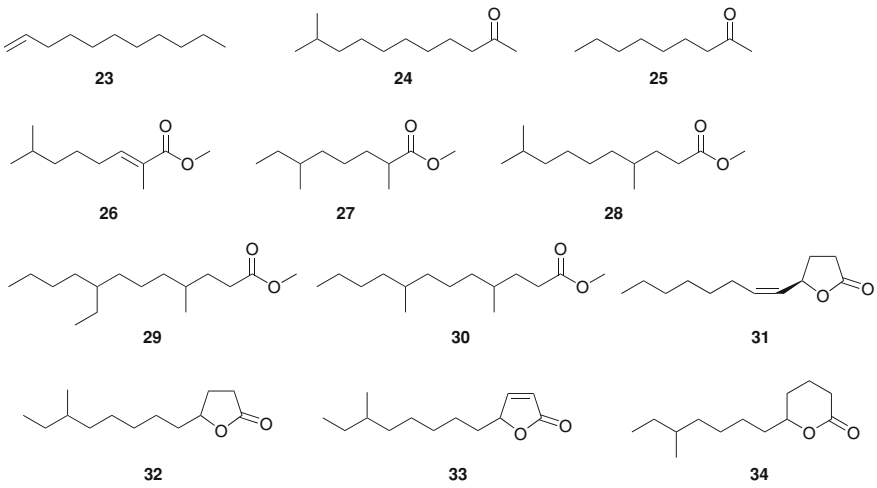

Fig. 3.5 Various VOCs derived from the fatty acid biosynthetic pathway

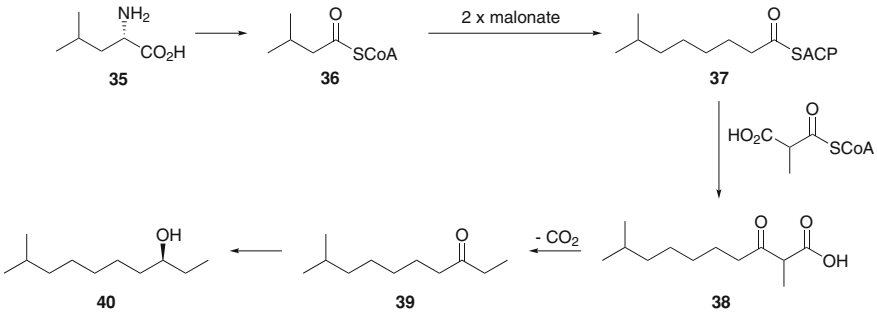

Fig. 3.6 Biosynthesis of the volatiles 9-methyl-3-decanone (**39**) and (*S*)-9-methyldecan-3-ol (**40**) of *Myxococcus xanthus*

to variation of the chain length of derived volatiles (Bode et al. 2005). Ketones of this type are relatively widespread found in many different bacterial groups. Simple methyl ketones such as 2-nonanone (**25**) are common compounds (Lemfack et al. 2018), present in different bacteria. In contrast, **39** and the related alcohol (*S*)-9-methyldecan-3-ol (**40**) are singular compounds, specific for *M. xanthus* (Dickschat et al. 2004).

Although methyl esters of unbranched acids are released in many bacteria (Lemfack et al. 2018), branched and unsaturated derivatives are more specific. *Chitinophaga* Fx7914 produces more than 30 saturated and unsaturated methyl esters carrying a C-2 methyl group, such as methyl (*E*)-2,7-dimethyloct-2-enoate (**26**) and methyl 2,6-dimethyloctanoate (**27**). While the ester methyl group originates from *S*-adenosylmethionine (SAM), the C-2 methyl group originates from incorporation of propanoate (Nawrath et al. 2010a). Other branched esters are produced by

Micromonospora aurantiaca (Dickschat et al. 2011). A broad variety of methyl esters occurs with methyl groups at C-2 or C-4 and terminal methyl groups, such as methyl 4,8-dimethyldodecanoate (**30**) or methyl 4,9-dimethyldecanoate (**28**), but also saturated 2-methyl branched esters are known from *Chitinophaga.* An ethyl-branched ester, methyl 8-ethyl-4-methyldodecanoate (**29**), was also detected. Feeding experiments confirmed that the biosynthesis follows the rules laid out in Fig. 3.4.

Similar as discussed in the previous chapter, oxidation at specific positions and ring closure can lead to lactones. Lactones are a group of compounds often used to transmit signals (Schulz and Hötling 2015). (4*R*,5*Z*)-Dodec-5-en-4-olide (**13**) is produced as major compound by *Loktanella* sp. (Dickschat et al. 2005b), together with 10-methyldodecan-4-olide (**32**), 10-methyldodec-2-en-4-olide (**33**), and 10-methyldodecan-5-olide (**34**), the latter also being emitted by *Streptomyces* sp. (Citron et al. 2015, 2012a).

Fatty acids can also be converted into aliphatic compounds carrying rarer functional groups, such as nitriles. Fatty acid derived nitriles have so far only been reported as volatiles of *Pseudomonas veronii* and *Micromonospora echinospora* (Montes Vidal et al. 2017). Feeding studies with stable isotopes verified that the biosynthesis of the nitriles differs in the two strains. Common fatty acids are converted into saturated (**50**) and unsaturated (**47**, **51**) C_{16}- and C_{18}-cyanides starting with typical bacterial fatty acids, such as stearic or vaccenic acids, via transformation into the respective amides, followed by dehydration. Unsaturated acids are synthesized by dehydration of the 3-hydroxydecanoate intermediate of the fatty acid biosynthesis to 2-decenoate (**41**) and isomerization. The resulting intermediate, (*Z*)-3-decenoate (**43**), is differently used by *P. veroni* and *M. echinospora*. In the latter case no further chain elongation as in *M. echinospora* is performed. The double bond stays at C-3, leading to shorter alkyl cyanides **47** (Fig. 3.7). In case of *P. veroni* continuing elongation of **43** leads to ω-7 unsaturated C_{16}- and C_{18}-nitriles (**51**).

Fatty acids can also be part of volatile compounds that are fused with other precursors. Salinilactones are volatile variants of the A-factor, a non-volatile signalling compound from *Streptomyces griseus*. The biosynthetic gene AfsA of *S. tropica* is responsible for biosynthesis of several salinilactones, e.g. the major one salinilactone B (**58**). It is likely formed by esterification of 3-oxoheptanoic acid (**53**) that is esterified with dihydroxyacetone phosphate (**54**), a similar process occurring in A-factor biosynthesis (Kato et al. 2007) (Fig. 3.8). An aldol-reaction forms the lactone ring in **55** that is reduced to the phosphorylated lactone **56**. While a phosphatase would yield A-factor type compounds, proton abstraction leads to a stabilized anion **57** that can react internally forming the unique bicyclic lactone structure of **58** (Schlawis et al. 2018). The lactone **58** usually occurs with a range of analogs with shorter or longer side chain that are compounds so far only detected in *Salinispora*, while other A-factor producing bacteria lack them (Schlawis et al. 2020).

Polyketides are biosynthetically related to the fatty acids biosynthetic pathway. Surprisingly, only few volatiles are known to be polyketides. One example is streptopyridine (**62**) and related pyridines from *Streptomyces* sp. FORM5 (Groenhagen et al. 2014). Its origin from the polyketide pathway has been

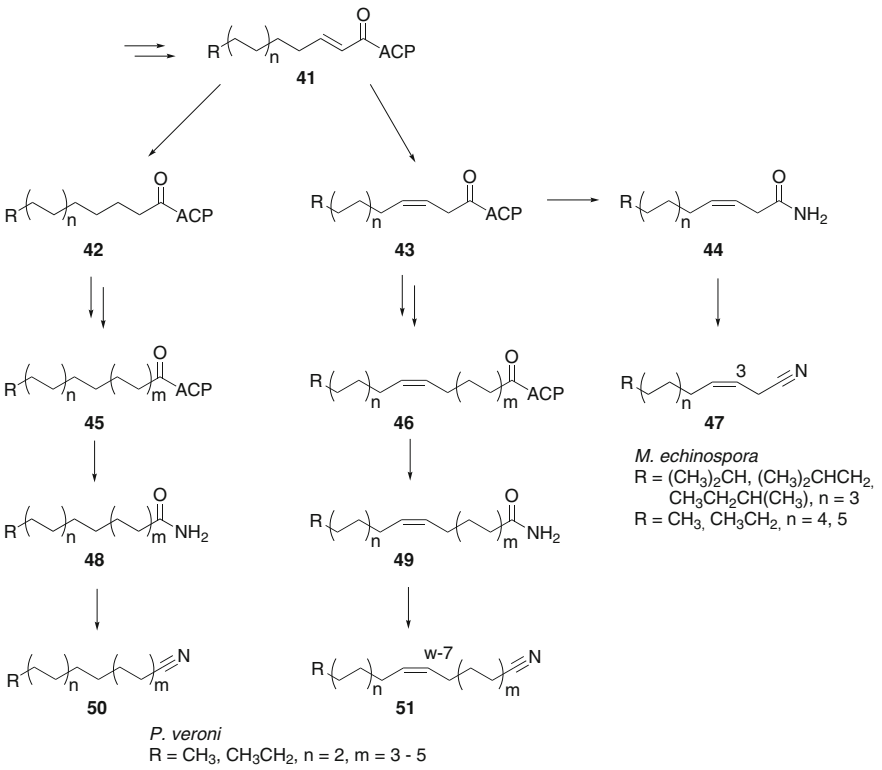

Fig. 3.7 Biosynthesis of aliphatic cyanides originating from the fatty acid biosynthetic pathway. The pathway is different in *Pseudomonas veronii* and *Micromonospora echinospora*

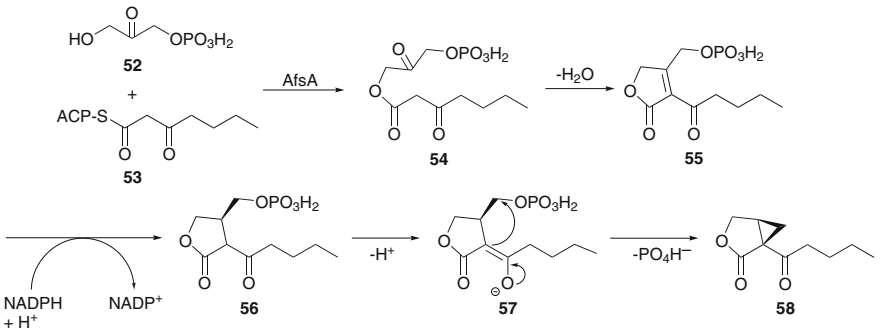

Fig. 3.8 Biosynthesis of salinilactones (**58**) in *Salinispora*

established by feeding of labeled acetate (Fig. 3.9). The antibiotic streptazoline is also produced by this strain, suggesting a connected biosynthesis. The streptazoline precursor **59** is converted by reduction and transamination into amine **60** that cyclizes to **61**. Elimination of water and oxidation delivers **62**. Interestingly,

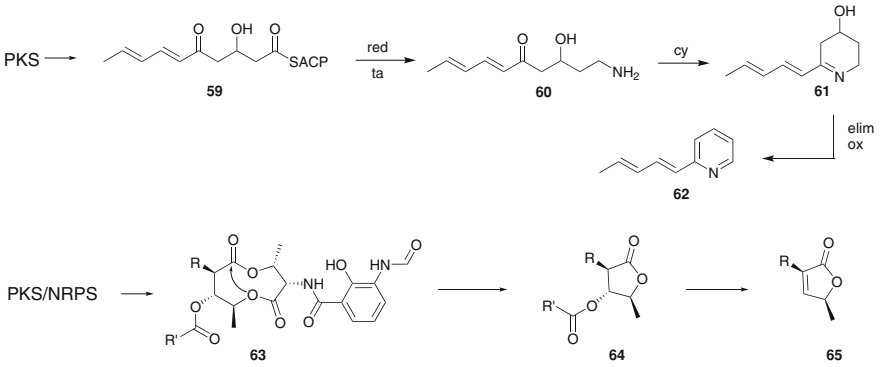

Fig. 3.9 Polyketide derived VOCs. A polyketide synthase (PKS) forms the streptazolin precursor **59**. Reduction (red) and transamination (ta) deliver the intermediate **61** that is further modified by cyclization (cy), elimination (elim) and oxidation (ox) to furnish streptopyridine (**62**). The PKS part of the antibiotic antimycin (**63**) can release blastmycinones (**64**) by transesterification. Elimination of the carboxylic acid delivers the butenolides **65**. The alkyl residues R and R′ range from C_1 to C_7, including methyl and ethyl branches

hydrogenated analogs with an NH group, e.g., **61**, were detected in solvent extracts of the liquid medium cultures, but not in the headspace. This indicates that strong H-bond networks inhibit volatilization especially of amines.

Other unique lactones are blastmycinones and related butenolides, **64** and **65**, produced by various streptomycetes (Riclea et al. 2012). They are derived from the antibiotic antimycin that is formed by a hybrid non-ribosomal peptide synthetase (NRPS)/polyketide synthase (PKS) (Yan et al. 2012; Sandy et al. 2012). Knock-out of the antimycin biosynthetic gene cluster also abolished production of the blastmycinones. About 30 different compounds were identified, released by antimycin producing streptomycetes (Riclea et al. 2012).

3.4 Aromatic Compounds

Another group of VOCs occurring often represent aromatic compounds, originating largely from the shikimate pathway. Some aromatic compounds are very common. Probably the most widespread compound among bacterial VOCs is 2-phenylethanol (**66**) that can be formed from phenylalanine or phenylpyruvate, usually by reduction of the intermediate phenylacetaldehyde (Fig. 3.10) (Liu et al. 2018). Indole (**72**) is also frequently encountered. Some bacteria can use a tryptophanase (Ren et al. 2004) to produce large amounts of indole, e.g., *Escherichia coli*, but **72** is also an intermediate in tryptophane biosynthesis (Lee and Lee 2010), thus explaining its occurrence even in bacteria lacking a tryptophanase, e.g., *Roseobacter* group bacteria and many others (Ziesche et al. 2015). Derivatives of the phenylpyruvate and related metabolism are occurring also quite often, e.g., methyl 2-phenylacetate (**67**),

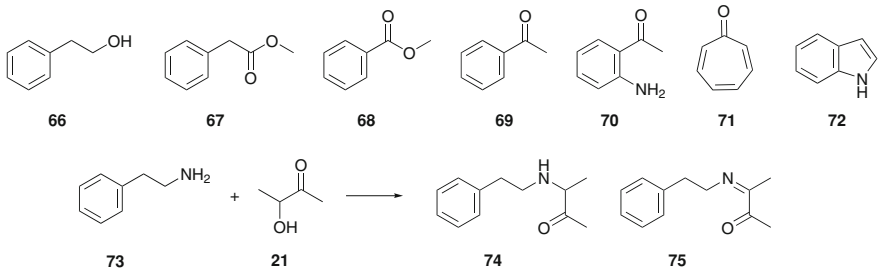

Fig. 3.10 Aromatic compounds found as volatiles emitted by bacteria. Compounds **66–69** and **72** are common compounds, released by many phylogenetically unrelated bacteria. The schleiferones (**74** and **75**) are synthesized non-enzymatically

methyl benzoate (**68**), or acetophenone (**69**) that is biosynthetically formed from benzoic acid, a product of phenylalanine degradation (Dickschat et al. 2005c). Amino groups can be found in aromatic compounds as well, e.g., 2-aminoacetophenone (**70**), present in *Burkholderia* and *Pseudomonas*. It is a side product of the biosynthetic pathway leading to the dihydroquinolone quorum-sensing signals of these bacteria (Drees et al. 2016).

Another metabolite of the phenylacetate metabolic pathway is tropone (**71**) (Thiel et al. 2010). Its biosynthesis starts by epoxidation and ring cleavage of the aromatic ring system in 2-phenylacetate, followed by internal aldol condensation to form the carbocyclic seven-membered ring. Dehydration and decarboxylation furnishes **72** (Brock et al. 2014a).

If bacterial VOCs are reactive enough, they can react in a non-enzymatic reaction to form new compounds, sometimes with interesting bioactivity. The schleiferones A (**74**) and B (**75**) are reaction products of 2-phenylethylamine (**73**) and acetoin (**21**). They are reported from *Staphylococcus schleiferi* that releases both starting materials (Lemfack et al. 2016), but can also be formed from two independent producers, one releasing **21** and the other **73** (Kai et al. 2007).

Unique cyclohexenyl derivatives are used by *S. tropica*. The esters **88**, **92**, and **93** are major components of the volatile bouquet, next to methyl 2-phenylacetate (**67**) and methyl benzoate (**68**) (Groenhagen et al. 2016). They are formed via the shikimate pathway and are side products of the biosynthesis of cyclohexenylalanine, an amino acid needed for the biosynthesis of the proteasome inhibitor salinosporamide. Deletion mutant analysis revealed the close connection of the biosynthesis of these compounds, while **67** and **68** are independently formed. Details can be found in Fig. 3.11.

A combination of the fatty acid pathway and aromatic compounds leads to phenones such as 1-phenyldecan-1-one (**101**), produced by the myxobacterium *Stigmatella aurantiaca* (Dickschat et al. 2005c). Labeling studies showed that palmitic acid (**95**) is chain shortened to decanoic acid (**97**) and activated to form octylmalonyl-CoA (**99**). Reaction with benzoic acid, likely activated as CoA-ester (**98**) and obtained from phenylalanine (**94**), furnishes β-keto acid **100** that is decarboxylated to **101** (Fig. 3.12).

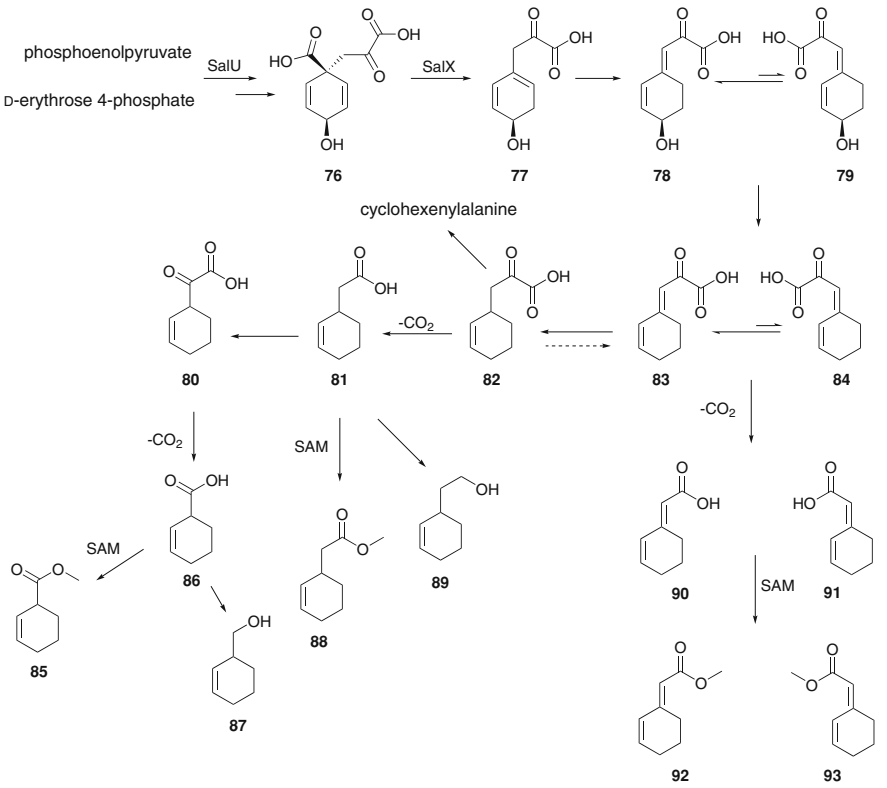

Fig. 3.11 Biosynthesis of cyclohexenyl derivatives of *Salinispora tropica* (Groenhagen et al. 2016). The biosynthesis of cyclohexenylalanine uses the ketocarboxylic acids **83** and **84** as intermediates. Decarboxylation leads to acids **90** and **91** that are methylated by SAM to major volatiles **92** and **93**. After hydrogenation and loss of CO_2, acid **81** is formed, serving as precursor of the major volatile **88** and various minor side products

3.5 Pyrazines

As shown above, fermentation is common in many bacteria, often leading to α-difunctionalized acetoins, diketones, aminoaldehydes, or aminoketones that are toxic. They have to be eliminated directly or can react to other compounds, e.g., pyrazines. Therefore, pyrazines can be found in many bacteria produced by condensation reactions (Dickschat et al. 2010a; Nawrath et al. 2010b; Kretsch et al. 2018). Pyrazines are sometimes released as a dominating compound (Beck et al. 2003), occur in complex mixtures (Dickschat et al. 2005d; Bañeras et al. 2013; Brunetti et al. 2019), but are also found as trace components in various strains. Their analysis is complicated by the observation that especially methylpyrazines are formed during autoclavation of bacterial growth media (DeMilo et al. 1996; Robacker et al. 1998).

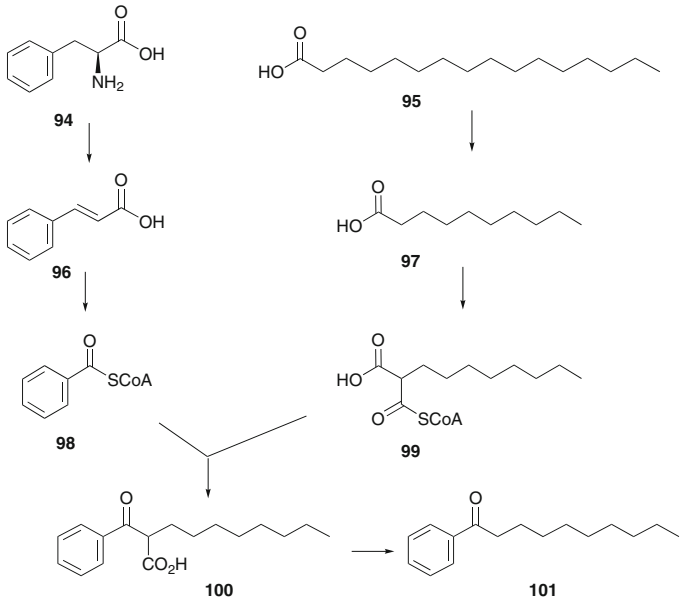

Fig. 3.12 Biosynthesis of 1-phenyl-2-decanone (**101**)

Three subtypes of pyrazines can be discerned, those substituted with up to four methyl or ethyl groups, those carrying one or two longer side chains, and finally methoxylated pyrazines. The first type, simple methyl or ethyl pyrazines such as tetramethylpyrazine (**104**), is biosynthesized by condensation of aminoketone and aminoaldehyde intermediates derived from acetoin (**21**). Transamination can occur to form an aminoalcohol that is oxidized to aminoketone **102** (Fig. 3.13). Alternatively, oxidation of **21** leads first to diacetyl that is transformed into **102** by transamination (Dickschat et al. 2010a). The aminoalcohols spontaneously dimerize to dihydropyrazines such as **103** that are instable in the presence of oxygen and readily form pyrazines, **104** in this case.

Similarly, higher dialkylpyrazines, e.g., 2,5-diisopropylpyrazine (**108**), are biosynthesized from the respective aminoaldehydes of aliphatic amino acids such as valine (**105**) (Fig. 3.13). Labeling studies showed that a cyclic dipeptide of the respective amino acids is not a precursor (Nawrath et al. 2010b). Recently, a biosynthetic gene cluster for a related compound, the non-volatile bis-*N*-oxide of **108**, was detected in *Pseudomonas* (Kretsch et al. 2018), indicating that the dimerization of aminoaldehydes can be enzyme mediated. During the condensation process rearrangements can occur, so that both **108** and its isomer 2,6-diisopropylpyrazine (**109**) are found in the same strain (Nawrath et al. 2010b).

Finally, methoxypyrazines such as 2-isopropyl-3-methoxypyrazine (**118**) are reported from various bacteria (Brunetti et al. 2019; Schulz et al. 2004). It has been proposed by labeling studies with ^{13}C-pyruvate isotopomers that methoxypyrazine **118** is synthesized by condensation of glycine (**110**) and valine

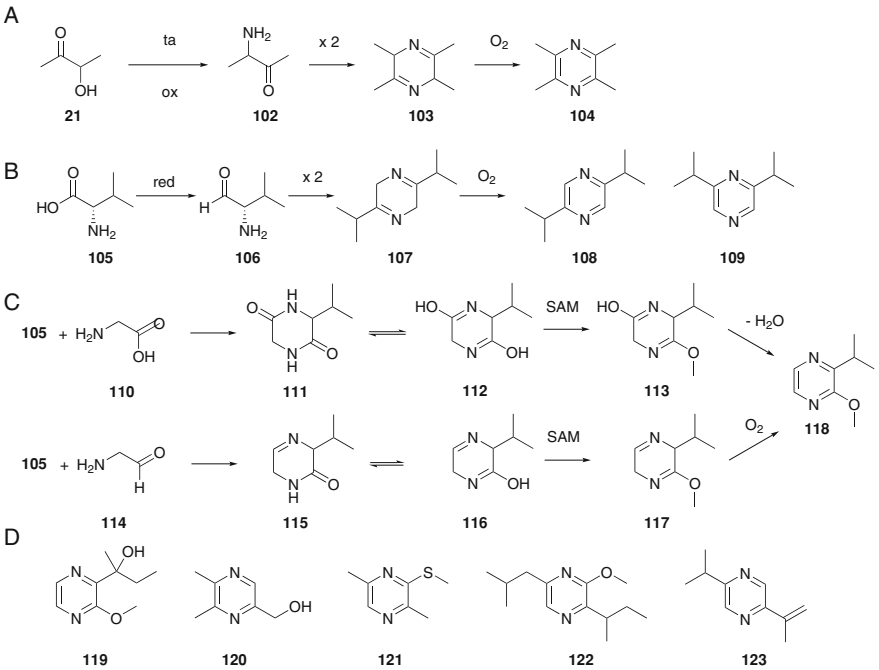

Fig. 3.13 Biosynthesis of methylpyrazines (**a**), higher alkylpyrazines (**b**), and methoxypyrazines (**c**). While the upper pathway in C was originally proposed, newer results favor the lower route. (**d**) Modified pyrazines

(**105**). The cyclic dipeptide precursor is then methylated by *S*-adenosylmethionine (SAM). In the last step, elimination of water furnishes the pyrazine ring (Cheng et al. 1991; Zhao et al. 2019). In light of the newer results it seems more likely that an amino acid, **105**, is condensed with an aminoaldehyde, e.g., **114**. SAM-mediated methylation might take place after condensation on **116**, or before at the amino acid stage (Fig. 3.13c). Compounds derived from two aliphatic amino acids such as **122** are also found (Dickschat et al. 2005d; Schulz et al. 2004).

Sometimes additional modifications of these pyrazines occur, e.g., hydroxylations in the side chains and eliminations, e.g., in 2-(1-hydroxy-1-methylpropyl)-3-methoxypyrazine (**119**) and 2-isopropyl-5-(propen-2-yl)pyrazine (**123**) from *Chondromyces crocatus* (Schulz et al. 2004), 2-(hydroxymethyl)-5,6-dimethylpyrazine (**120**) from *Corynebacterium glutamicum* (Dickschat et al. 2010a), and 2,5-dimethyl-3-(methylsulfanyl)pyrazine (**121**) from *Sulfitobacter* (Dickschat et al. 2005d) (Fig. 3.13d).

3.6 Sulfur Compounds

Sulfur compounds are also common microbial volatiles. They are mainly derived from H_2S and methanethiol. Methanethiol spontaneously dimerizes in air oxidatively to dimethyl disulfide (**135**) (Brock et al. 2014b; Bentley and Chasteen 2004). This common disulfide is much easier to detect and therefore more often reported than methanethiol. Other sulfur compounds, such as dimethyl trisulfide or *S*-methyl esters, e.g., *S*-methyl phenylethanethioate (**136**), are also derived from methanethiol (Brock et al. 2013a; Dickschat et al. 2010b). The metabolism of methionine and cysteine leads to a variety of short chain compounds carrying sulfur substituents such as 3-(methylthio)propanal (**137**) or 4-methylthiobutan-2-one (**138**) (Schulz and Dickschat 2007; Groenhagen et al. 2013). More elaborated sulfur compounds also exist, e.g., 2-methyltetrahydrothiophen-3-one (**130**). Labeling studies revealed that **130** is formed by condensation of homocysteine and pyruvate (Nawrath et al. 2010c) (Fig. 3.14). Pyruvate (**128**) is activated under loss of CO_2 with thiamine to form the ketol **133**, similar to acetoin (**21**) formation. This adduct reacts with 3-thiopropanal (**126**) that is formed from homocysteine (**124**) by transamination and loss of CO_2. Hemithioacetal formation of the sulfur group then leads to enol (**131**) that can tautomerize to target ketone **130**, while reduction or oxidation leads to minor by-products, **127** or **134**.

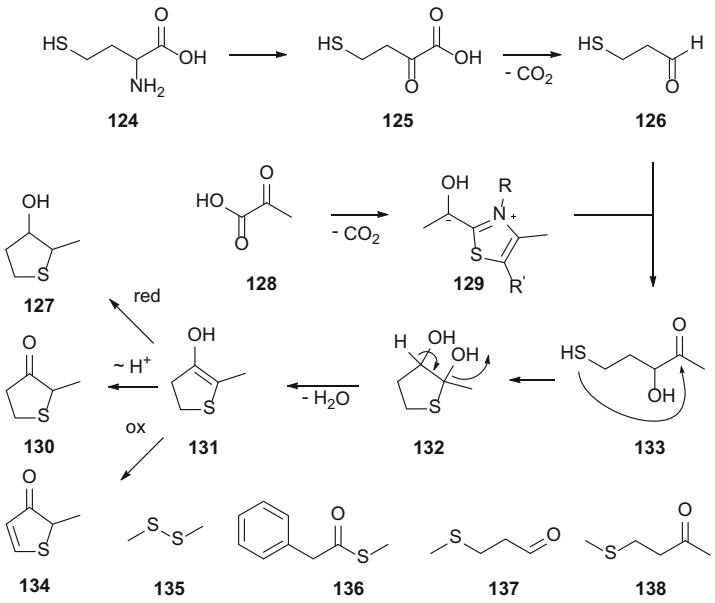

Fig. 3.14 Biosynthesis of cyclic sulfur derivatives via acetoin-type intermediate **133** and other sulfur derivatives

3.7 Terpenes

Volatiles derived from the terpene biosynthetic pathway represent the structurally most diverse group of bacterial VOCs. While earlier mostly plants and fungi were associated with the biosynthesis of terpenes, it became evident that bacteria are also promiscuous producers of such compounds. Volatile terpenes are mostly hydrocarbons and alcohols, but also ethers or ketones and epoxides occur. Sesquiterpenes, C_{15}-compounds, dominate, but monoterpenes (C_{10}) or diterpenes (C_{20}) are produced as well. In addition, degraded or modified terpenes, the terpenoids, containing less or more carbons than the respective parent compounds, are formed by special enzymes in many bacteria. Different to the other compound classes, the enzymes responsible for bacterial terpene production have been characterized in many cases and the biosynthetic pathway to these compounds has been elucidated, often in detail (Yamada et al. 2015; Dickschat 2016; Citron and Dickschat 2014). Furthermore, the knowledge on the biosynthetic gene clusters of terpene synthases allows the use of online tools such as ANTISMASH to locate terpene synthases in genomes of sequenced bacteria (Blin et al. 2019). Nevertheless, the structure of the produced terpenes cannot be deduced by genomic analysis.

Only few monoterpenes have been characterized from bacteria, e.g., linalool (**152**), the structurally simplest monoterpene alcohol, or 1,8-cineol (**151**), for which the synthase from *Streptomyces clavuligerus* has been isolated and the biosynthetic pathway is elucidated (Rinkel et al. 2016).

Sesquiterpenes are biosynthesized from the universal precursor farnesyl pyrophosphate (**135**) that is formed either via the mevalonate or the desoxyxylulose pathway, both operating in bacteria. A typical pathway to bacterial sesquiterpene hydrocarbons and alcohols exhibiting a cadinane backbone is shown in Fig. 3.15 (Citron et al. 2015). The pyrophosphate **135** is converted into nerolidyl pyrophosphate (**136**) to allow cyclization via cationic intermediates, typical for terpene chemistry. The following 1,10-cyclization and H-shift yields the monocyclic germacryl-cation **138** that can cyclize a second time to form cadinane-type carbocycles. Depending on the enzymes, different stereochemistry in the ring junction divides the pathway. While the *trans*-cation **140** can be trapped by water to form α-cadinol (**139**), the *cis*-cation **143** can lose a proton to form the hydrocarbons α-muurolene (**141**) and δ-cadinene (**142**). Besides of trapping of water or elimination of protons, 1,x-hydride shifts are common reactions, allowing access to new structures. An 1,2-hydride shift from **143** to **146** opens the pathway to cadina-1,4-diene (**144**) and *trans*-cadina-1(6),4-diene (**145**), while trapping with water delivers *epi*-cubenol (**150**). Further hydride shifts allow access to zonarene (**148**) and cadina-3,5-diene (**149**).

These and other sequiterpenes have been detected in various actinomycetes (Citron et al. 2015), often with characterized synthases. Such compounds include *epi*-cubebol (**159**) (*Streptosporangium roseum*) (Dickschat et al. 2014), *epi*-cubenol (**150**) (*S. griseus*) (Nakano et al. 2012; Cane et al. 1993), δ-cadinene (**142**) (*S. clavuligerus*) (Hu et al. 2011), γ-cadinene (*Chitinophaga pinensis*, a

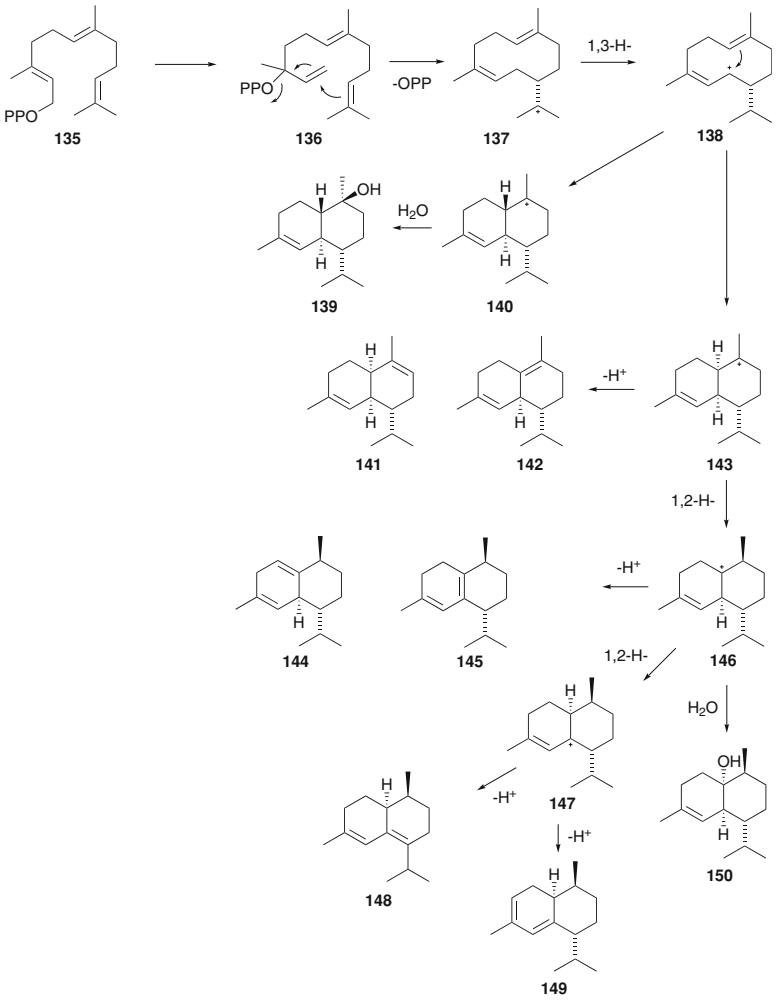

Fig. 3.15 Biosynthesis of cadinane-type sesquiterpenes

Bacteroidetes) (Rabe and Dickschat 2013), τ-muurolol (**156**) (*S. clavuligerus*, *Roseiflexus castenholzii*) (Hu et al. 2011; Rabe and Dickschat 2013; Yamada et al. 2015), selina-4(15),7(11)-diene (**155**) (*S. pristinaespiralis*) (Baer et al. 2014) and pentalenene (**160**) (Cane et al. 1990; Quaderer et al. 2006) (Fig. 3.16). New sesquiterpenes not known, e.g., from plants include (−)-neomeranol B (**162**), (+)-intermedeol (**163**) accompanied by known (+)-isodauc-8-en-11-ol (**164**). The enzymes from three *Streptomyces* species were characterized and the biosynthetic pathway was elucidated using isotope labeling (Rabe et al. 2016a). Similarly, (+)-isoafricanol (**163**) is the major product of a sesquiterpene synthase from

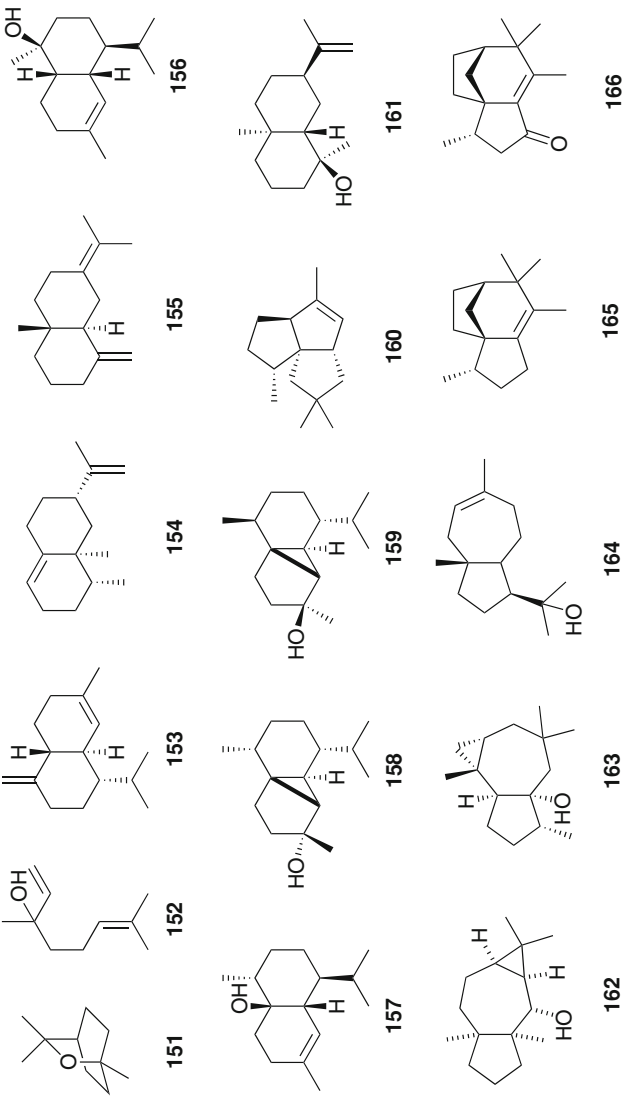

Fig. 3.16 Volatile terpenes released by actinomycetes and myxobacteria

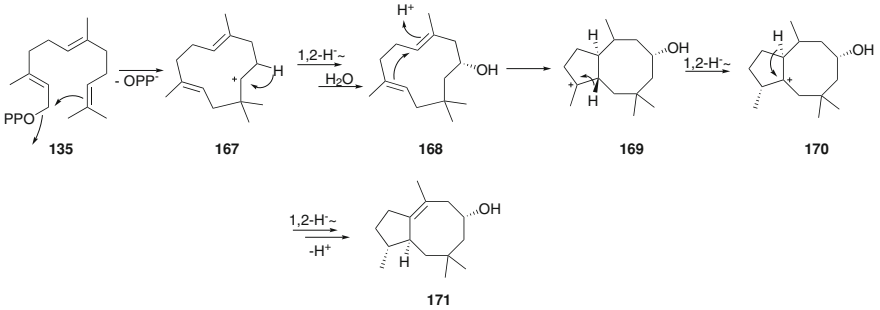

Fig. 3.17 Biosynthesis of pristinol (**171**)

S. malaysiensis (Rabe et al. 2017a*;* Riclea et al. 2014) and (−)-7-*epi*-α-eudesmol (**21**) of a terpene cyclase from *S. viridochromogenes* (Rabe et al. 2016b).

The biosynthesis of pristinol (**171**) (Fig. 3.17), showing an unusual 5/8-ring skeleton, is an example that not only hydrocarbons can undergo a second ring closure to form more elaborate structures (Klapschinski et al. 2016). Here, the first formed sesquiterpene alcohol, (*R*)-hyemalol (**168**), is again protonated to form the bicyclic system. A couple of 1,2-hydride shifts finally lead to **171**.

But also other families are prolific producers of sesquiterpenes, e.g., myxobacteria. *Sorangium cellulosum* harbors a synthase specifically producing (+)-eremophilene (**154**) (Schifrin et al. 2015) and another, less specific one producing 17 different sesquiterpenes, including the major compounds, tricyclic 10-epi-cubebol (**158**), a diastereomer of **159**, and δ-cadinene (**142**) (Schifrin et al. 2016).

Besides common hydrocarbons and alcohols, other functional groups are also found in the bacterial sesquiterpenes. These include ethers, exemplified by **151** or the corvol ethers **175** and **176**, produced by the actinomycete *Kitasatospora setae* (Rabe et al. 2015). Detailed studies with labeled precursors showed that the heterologously expressed corvol ether synthase converts farnesyl diphosphate (**135**) first into germacrene D-4-ol (**172**) (Fig. 3.18). Cyclisation is induced by protonation at C-5, followed by two sequential 1,2-hydride shifts leading to cation **174**. While another 1,2-hydride shift and an intramolecular attack of the hydroxy-group lead to corvol ether A (**176**), a Wagner-Meerwein rearrangement and capture of the neighboring hydroxy-group furnishes corvol ether B (**175**). This detailed investigation showed that neutral sesquiterpenes such as **172** can serve as precursor for further modifications leading to even higher structural diversity.

Ketone containing sesquiterpenes are less common. An prominent example is albaflavenone (**166**), produced by *Streptomyces coelicolor* A3(2) (Gürtler et al. 1994), but also by other streptomycetes (Moody et al. 2012). The hydrocarbon *epi*-isozizaene (**165**) is oxidized by a cytochrome P 450 oxidizing enzyme to form **166** via the respective alcohols (Zhao et al. 2008).

While many compounds discussed here are only reported so far from a specific or few strains, the wide distribution of sesquiterpene synthases within the

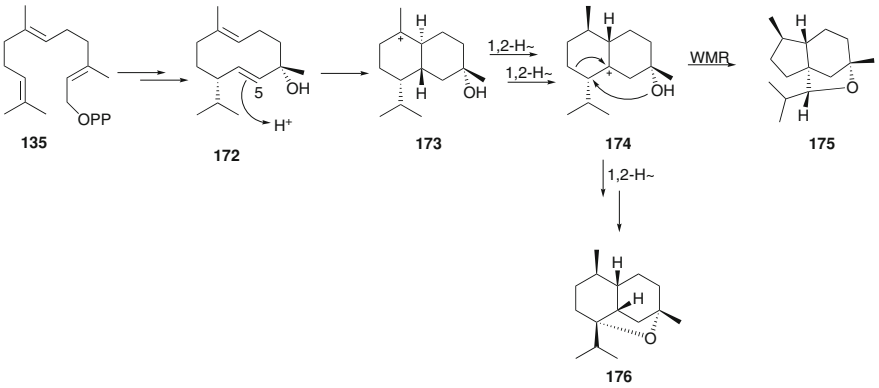

Fig. 3.18 Biosynthesis of the corvol ethers A (**176**) and B (**175**). *WMR* Wagner-Meerwein-rearrangement

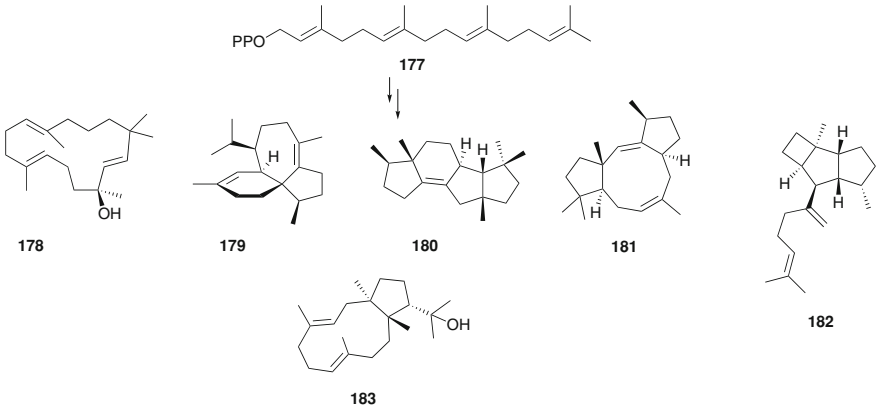

Fig. 3.19 Some bacterial diterpenes from actinomycetes and bacteroidetes

actinomycetes points not towards a singular occurrence of a certain compound. Instead a group specific distribution can be assumed. This is further underlined by successful genetic approaches to link terpene synthase gene phylogeny to specific products (Dickschat 2016).

Diterpenes can also be detected by headspace methods, thus ensuring their transport through the gas phase. Because much of the material may not be volatilized, the small peaks usually observed for diterpenes in headspace GC/MS indicate a potentially much higher concentration in the bacterium itself. Only recently it became clear that bacterial diterpenes might be structurally as diverse as sesquiterpenes (Yamada et al. 2015; Dickschat 2019). Geranylgeranyl pyrophosphate (**177**) serves as starting material, similar as **135** for sesquiterpenes (Fig. 3.19). Examples are micromonocyclol (**178**) from *Micromonospora marina* (Rinkel and Dickschat

2019), bonnadiene from *Allokutzneria albata* (**179**) (Lauterbach et al. 2018), cattleyene (**180**) from *S. cattleya* (Rinkel et al. 2019), tsukubadiene (**181**) from *S. tsukubaensis* (Yamada et al. 2015; Rabe et al. 2017b), or prenylkelsoene (**182**) from *S. xinghaiensis* (Rinkel et al. 2017). Outside the actinomycetes, *Chitinophaga pinensis* (Bacteroidetes) also possesses a diterpene synthase responsible for the production of 18-hydroxydolabella-3,7-diene (**183**) (Dickschat et al. 2017).

3.8 Modified Terpenes

Regular terpenes discussed so far are found in different kingdoms. In contrast, some bacteria have developed modified pathways that are more or less specific for them. These can include additional methyl groups, as found in the homomonoterpene 2-methylisoborneol (**190**) (Brock et al. 2013b; Gerber 1969) and the methylated sesquiterpene sodorifen (von Reuß et al. 2010, 2018), while geosmin is a degraded sesquiterpene (Jiang et al. 2007; Gerber 1968).

The biosynthesis of 2-methylisoborneol has been investigated in detail (Fig. 3.20) (Dickschat et al. 2007) and the respective enzymes characterized (Wang et al. 2011; Wang and Cane 2008). Geranyl pyrophosphate (**184**) is first methylated by SAM leading via **185** to methylgeranyl/methyllinalyl pyrophosphate (**186**, **187**). Cyclization leads to cation **188** that can undergo a second cyclization to form **189**. Trapping by water leads to the earthy smelling **190**. The intermediate **187** can also follow known monoterpene formation pathways leading, e.g., to linalool, fenchol, or

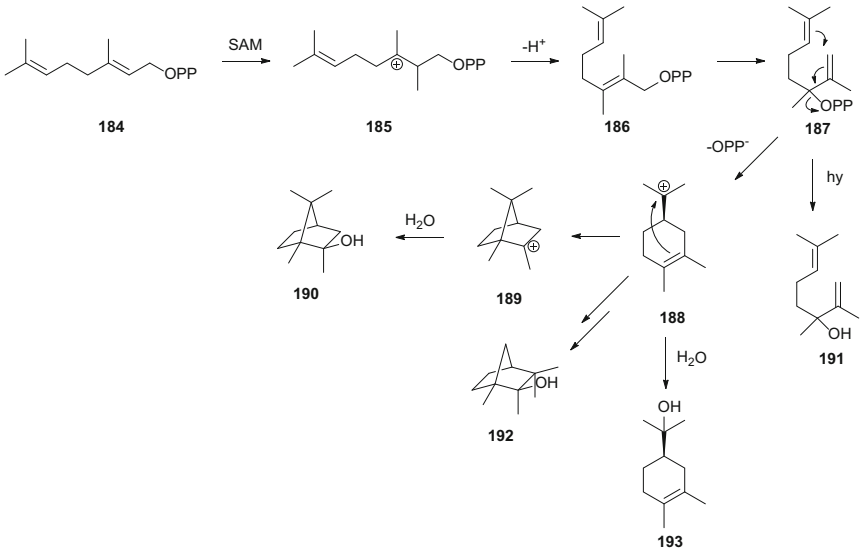

Fig. 3.20 Biosynthesis of 2-methylisoborneol (**190**) and other methylated monoterpenes. *hy* hydrolysis

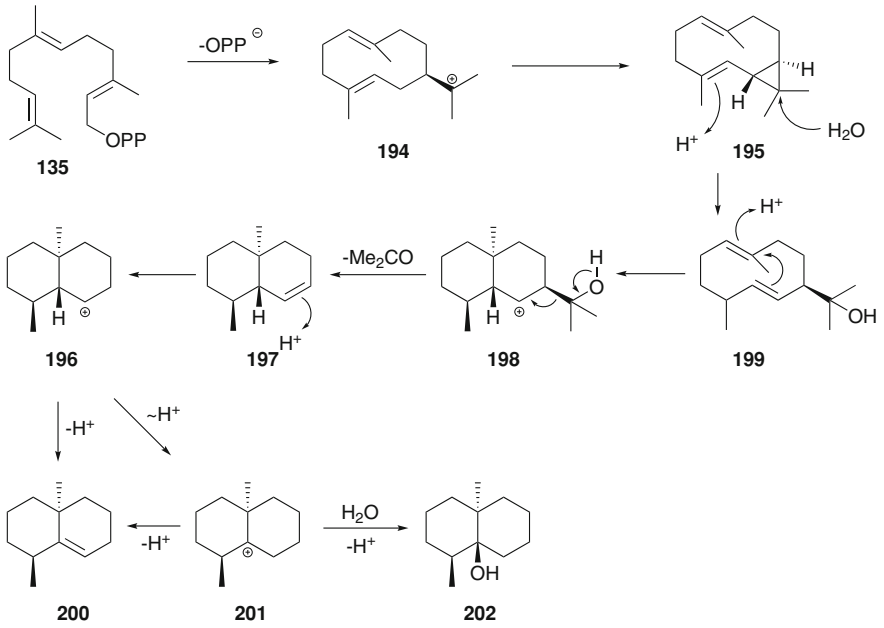

Fig. 3.21 Biosynthesis of geosmin (**202**)

terpineol. This explains the detection of the methylated analogs methyllinalool (**191**), methylfenchol (**192**), and methylterpineol (**193**) in headspace analyses (Brock et al. 2013b). 2-Methylisoborneol (**190**) is released by many actinobacteria, myxobacteria, and cyanobacteria (Schulz and Dickschat 2007; Jüttner 1995; Watson et al. 2016).

Even more widespread occurring than **190** is geosmin (**202**), a group compound of actinobacteria (Citron et al. 2012b; Yamada et al. 2015), myxobacteria (Dickschat et al. 2005e), and cyanobacteria (Schulz and Dickschat 2007; Jüttner 1995). Its biosynthesis is shown in Fig. 3.21 (Dickschat et al. 2005e). Farnesyl pyrophosphate (**135**) is first cyclized to the germacradienyl-cation **194**. β-H-Abstraction likely leads to isolepidozene (**195**) that is immediately protonated and under ring-opening water addition delivers germacradienol (**199**) (Jiang et al. 2007). This sesquiterpene usually co-occurs with geosmin and serves as its precursor. The geosmin synthase is a bifunctional enzyme, one part being responsible for the formation of **199**, while the other furnishes finally **202** (Jiang et al. 2007). In this second part **199** is protonated and cyclizes to form **198**, followed by a loss of acetone in a Prins-type reaction to form octalin **197** (Nawrath et al. 2008; Dickschat et al. 2005e). Again protonation, followed by an 1,2-hydrogen shift and addition of water, delivers geosmin (**202**) (Dickschat et al. 2005e). Intermediate **196** can also form octalin **200** as a byproduct by proton abstraction (Nawrath et al. 2008).

The rhizobacterium *Serratia plymuthica* releases a methylated sesquiterpene, sodorifen (**215**), that in contrast to group compounds 1**90** and **202** seems to be

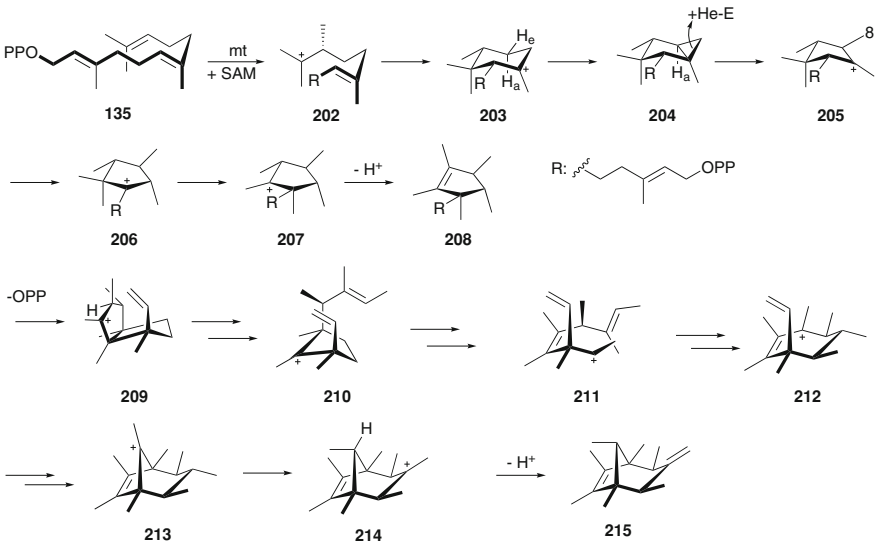

Fig. 3.22 Proposed biosynthetic pathway to sodorifen (**215**)

specific for this bacterium (von Reuß et al. 2010). The postulated biosynthetic pathway to **215** starts from **135**, generated via the desoxyxylulose pathway (von Reuß et al. 2010), first producing the precursor pre-sodorifen pyrophosphate (**208**) (Fig. 3.22). A methyltransferase methylates the double bond at C-10 of **135**, generating cation **202**. Formation of a cyclopropane ring, followed by reprotonation, induces ring contraction to **205**. Hydride and methyl-shifts follow, establishing the pre-sodorifen pyrophosphate intermediate **208**. Loss of the pyrophosphate group induces a complicated cascade including extensive hydride and methyl group shifts. Together with ring closing, fission, and expansion processes they lead via the bicyclic intermediate **209** and the ring opened compound **210** und **211** to the cycloheptenyl cation **212**, the precursor of the final ring closure. A 1,4-H-shift on **213** gives the sodorifen-precursor cation **214** and loss of a proton generates sodorifen (2**15**). It seems likely that many intermediates might serve as precursors of other, structurally not characterized methylated sesquiterpenes in this strain (von Reuß et al. 2018).

3.9 Summary

In summary, volatiles of bacteria are structurally highly diverse. While some can be regarded as by-products of primary metabolism, others need a more elaborate biosynthetic machinery. In some cases their formation is linked to other metabolites of the producing strain, but independent pathways have also been evolved. Usually mixtures of compounds are released, with widely varying concentrations. Their

effects on other organisms and their biosyntheses need to be investigated in more detail in the future, hopefully reaching an integrated picture of bacterial volatile chemical ecology.

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Chapter 4
In Vivo and In Vitro Volatile Organic Compounds (VOCs) Analysis in Bacterial Diagnostics: Case Studies in Agriculture and Human Diseases

Moamen M. Elmassry and Mohamed A. Farag

Abstract With an increasing interest in volatiles metabolic pathways within bacterial species, investigation into the role of volatile organic compounds (VOCs) produced by different bacteria and their potential application in diagnostics now follows. A significant body of work was accumulated over the past few decades to achieve such goal, mostly aided by the advances in volatiles analytical detection technologies. A handful of pathogenic bacterial species have been extensively investigated and potential VOCs biomarkers or profiles have been proposed to diagnose such pathogens during infection. However, it was found that developing species-specific VOCs biomarkers is much more challenging than just VOCs profile characterization. Using VOCs profile fingerprinting for pathogen detection is promising without the need to identify exact VOCs. Studying VOCs produced in vivo and in vitro by the same species additionally showed discrepancy between the two experimental setups, which is a result of the different growth conditions, nutrients utilization, and background VOCs produced from the culture medium or the host. Such discrepancy has hindered the translation of numerous in vitro results to in vivo studies. Consequently, in this chapter we review results from in vitro versus in vivo experimental setups in the context of two major applications including bacterial VOCs diagnostics in agriculture and human diseases.

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Abbreviations

APCI	Atmospheric pressure chemical ionization
CF	Cystic fibrosis
eNose	Electronic nose
GC	Gas chromatography
MS	Mass spectrometry
PGPR	Plant growth-promoting rhizobacteria
SIFT	Selected ion flow tube
SPME	Headspace solid-phase microextraction
VOCs	Volatile organic compounds

4.1 Introduction

Microscopic organisms as bacteria encompass diverse metabolic pathways that yield a unique combination of metabolites including thousands of volatiles that serve numerous functions, from signaling to pathogenesis (Bos et al. 2013; Rees et al. 2018). As a result of genetic and metabolic, both anabolic and catabolic, diversity across bacterial species, it has been hypothesized that different bacterial taxa exhibit distinct volatiles fingerprint (Surette and Davies 2008; Lemfack et al. 2018). A great interest has been assumed towards the use of those species-specific volatile organic compounds (VOCs) to verify the presence or absence of certain microbial species (Rees et al. 2018). Such interest in the use of microbial volatiles as anchors to aid in its identification was augmented with piling evidence correlating human resident bacterial community (microbiome) with health and disease status (Human Microbiome Project Consortium 2012; Lynch and Pedersen 2016). The success of such approach involving identifying bacteria through their volatiles fingerprint has been eased owing to the recent advances in analytical methodologies for volatiles profiling in biological systems (Khalil et al. 2017; Rees et al. 2018). Beyond the achieved progress in elucidating the role of VOCs in microbial biology as airborne signals, further investigation of this poorly explored research area should now follow more towards the discovery of biomarkers of clinical or agricultural interest (Audrain et al. 2015).

The aim of this chapter is to provide a comprehensive overview on the applications of in vivo VOCs analysis to identify bacterial species commonly associated with agriculture and human diseases. Followed by, a discussion of the challenges for identifying VOCs in vivo recognizing limitations and needed future work. An outline of the review main theme and covered topics is presented in Fig. 4.1.

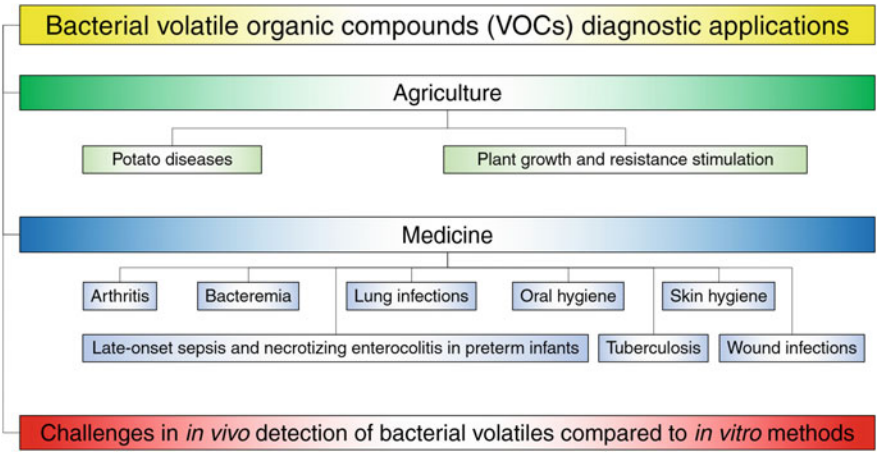

Fig. 4.1 An outline of the review theme to include advances in the research area that investigated VOCs associated with agriculture and human diseases and their application as diagnostic markers, and to highlight challenges facing identifying VOCs from in vivo and in vitro setups

4.2 Bacterial Volatiles Diagnostic Applications in Agriculture and Medicine

4.2.1 Agriculture

Agriculture is an indispensable part of human life. Plant diseases and resistance have been a major concern to maintain or even increase the productivity of agricultural production. Herein, we summarize the advances in using VOCs in identifying bacterial diseases in various plants and or the role of VOCs in stimulating plant growth and resistance.

4.2.1.1 Plant Diseases

Bacterial pathogens cause substantial economic losses worldwide by damaging valuable crops. Thus, there has been an urgent need for rapid and reliable diagnostic methods that can be used for crop disease detection at earlier stages to avoid for crop loss. This need has been partially fulfilled owing to the recent advances in detection technologies, but is far from perfect (Cui et al. 2018). In potatoes, two of the common diseases are potato brown and ring rot, which are caused by *Ralstonia solanacearum* and *Clavibacter michiganensis*, respectively. In vivo gas chromatography-mass spectrometry (GC-MS) analyses of infected potato tubers with either of these pathogens resulted in identifying disease related volatiles including 1-hepten-3-ol, 3,6-dimethyl-3-octanone, 3-ethyl-3-methylpentane, 1-chloroctane, and benzothiazole as markers of potato brown rot, whereas

2-propanol and toluene as markers of potato ring rot (Blasioli et al. 2014). Although, the origin of 1-chloroctane is an artifact and not from natural origin. In apple and pear plants, VOCs analysis using electronic nose (eNose) was successfully used to discriminate between non-infected plants and plants infected with *Erwinia amylovora* (fire blight) (Spinelli et al. 2010). Lastly, tobacco plants infected with different strains of *Pseudomonas syringae* induced changes in VOCs profiles that were dependent on the strain and its virulence (Huang et al. 2003). Despite the success in identifying specific VOCs to plant diseases inside labs, its integral to test the practicality of this approach in open fields that have high noise to signal ratio compared to in vitro setups.

4.2.1.2 Plant Growth and Resistance Stimulation

The microbiome associated with soil around plant roots (rhizosphere) is known for its function in stimulating plant growth, hence its name, plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1980) was derived. Since the discovery of the mechanistic beneficial effect of PGPR on plant growth that occurs through VOCs, this area of research flourished for its wide range of agricultural applications on important crops (Farag et al. 2013). This was first documented through in vitro VOCs exposure assays such as 2,3-butanediol and 3-hydroxy-2-butanone that are produced by *Bacillus subtilis* and *Bacillus amyloliquefaciens* and found to enhance the growth of *Arabidopsis thaliana* seedlings (Ryu et al. 2003). Furthermore, *Bacillus*-produced volatile, 2,3-butanediol, improved the defense response of *A. thaliana* seedlings to the bacterial pathogen, *Erwinia carotovora* (Ryu et al. 2004). A considerable body of work followed that finding positive interaction between plants and VOCs produced by plant-associated PGPR (Vespermann et al. 2007; Zhang et al. 2007; Meldau et al. 2013; Bailly et al. 2014). Recently, *Microbacterium* species was shown to be able to enhance *A. thaliana*, lettuce, and tomato growth through its sulfur volatile compounds (Cordovez et al. 2018). In the hope of elucidating the mechanism of plant growth promotion by bacterial VOCs, Tahir and colleagues found that *B. subtilis* VOCs promote tomato plant growth by triggering growth hormone activity (Tahir et al. 2017). The functioning of microbial volatiles in agricultural system as cues for either growth promotion induction and or priming defense responses in planta against pests or pathogens is an area that is still at its infancy.

VOCs have also been documented as important inter-kingdom signaling molecules (van Dam et al. 2016; Kai et al. 2016). For example, volatiles mostly esters and aromatics released from infected *Carex arenaria* roots were found to be able to recruit specific bacteria such as *Janthinobacterium*, *Collimonas*, and *Paenibacillus* (Schulz-Bohm et al. 2018). These recruited bacteria have antifungal properties and improve plant fitness upon biotic stress situations. Therefore, identifying VOCs responsible for attracting certain advantageous bacterial species over others is a promising area of applied research to develop more resistant plants (Bitas et al. 2013).

4.2.2 Human Diseases and Infections

In parallel to the increasing interest in how microbial volatiles act as signals in plant systems, a stronger interest in using microbial volatiles in the diagnosis of microbial infections in humans has been on the rise. Therefore, in this section we focus on several dozens of reports that investigated the potential application of bacterial volatiles detection in diagnosing arthritis, bacteremia, late-onset sepsis and necrotizing enterocolitis in preterm infants, lung infections, oral and skin hygiene, tuberculosis, and wound infections.

4.2.2.1 Arthritis

VOCs analysis was tested for its potential role in the differential diagnosis of arthritis. Analysis of the gas chromatograms of the synovial fluid collected from patients suffering from arthritis caused by trauma or pathogenic bacteria such as *Staphylococcus aureus*, *Streptococcus pyogenes*, or *Neisseria gonorrhoeae* showed distinct differences. These differences were attributed to accumulated metabolites from the metabolism of these pathogens or host metabolites that were further metabolized by the pathogens (Brooks et al. 1974). It was reported that the synovial lactate level could be used as diagnostic for arthritis with bacterial infection; however, other reports produced conflicting results due to variability and numerous confounding factors such as the causative pathogen and administration of antibiotics (Kirwan 1982; Edman and Brooks 1983).

4.2.2.2 Bacteremia

Bloodstream bacterial infection, or bacteremia, is a severe health risk to humans, which needs urgent care. Culturing techniques that are commonly used to identify bacterial pathogens behind bacteremia could delay appropriate diagnosis and treatment. This urged the development of novel approaches to diagnose patients with bacteremia. One approach that has been proven useful is atmospheric pressure chemical ionization mass spectrometry (APCI-MS). By studying volatile fingerprints from blood cultures infected with *S. aureus*, *Escherichia coli*, *Klebsiella pneumonia*, *Acinetobacter baumannii*, or *Pseudomonas aeruginosa*, Chingin and colleagues successfully used these fingerprints to diagnose bacteremia in patients. Advantages of this approach lies in its robustness and reliability. Prior to APCI-MS analysis, an incubation time of 16 h is required, which is shorter than traditional clinical and microbiological techniques typically needed for diagnosis. Moreover, such incubation time could be shortened by accelerating the bacterial growth in blood cultures by applying gentle agitation to incubated cultures. It is worth to note that fingerprinting approach does not require identifying the volatiles comprising the mass spectra; however, their chemical identity could prove useful for specificity in

its detection. Some of the identified biomarker signals include butyric acid and isovaleric acid *for S. aureus, indole for E. coli,* 1-vinyl aziridine for *P. aeruginosa,* and trimethylamine for *P. aeruginosa* and *A. baumannii* (Chingin et al. 2015). Such volatiles are not found normally in human blood, which confers specificity for each and its associated microorganism.

4.2.2.3 Late-Onset Sepsis and Necrotizing Enterocolitis in Preterm Infants

Preterm infant's population suffers high incidence and mortality rates of both necrotizing enterocolitis and late-onset sepsis. Unfortunately, pathophysiology of these diseases is still unclear (Bizzarro et al. 2005; Bentlin and de Souza Rugolo 2010; Berrington et al. 2012; Tröger et al. 2014; Patel et al. 2015). Unsurprisingly, changes in infants gut microbiome were associated with these diseases (Patel and Denning 2015; Rinninella et al. 2019). Consequently, more interest was given towards identifying gut and fecal VOCs produced by the gut microbiome to understand the pathogenesis and improve diagnosis of these diseases (Berkhout et al. 2018). Fecal VOCs profiling, based on eNose, of preterm infants with late-onset sepsis was successfully able to distinguish them from control 3 days prior to clinical symptoms onset (Berkhout et al. 2017). eNose proved useful in differentiating between infants with necrotizing enterocolitis based on the fecal VOCs profile 2–3 days prior to any clinical symptoms (de Meij et al. 2015). eNose presented a potential non-invasive tool that is clinically feasible for the early diagnosis of late-onset sepsis and necrotizing enterocolitis (de Meij et al. 2015; Berkhout et al. 2017). In another study, necrotizing enterocolitis diagnosis in preterm infants was associated with the disappearance of four esters: 2-ethylhexyl acetic ester, decanoic acid ethyl ester, dodecanoic acid ethyl ester, and hexadecanoic acid ethyl ester identified in their feces VOCs profile (Garner et al. 2009).

4.2.2.4 Lung Infections

Among the common infections affecting human is lung infections. Lung bacterial infections present the best model for assessing volatile microbial markers considering it can be detected directly in human breath without any intervention. Consequently, several reports have been made associating specific volatile marker in ventilator-associated pneumonia and cystic fibrosis lung infections to certain pathogens, for example, *P. aeruginosa*, *S. aureus*, and *A. baumannii.* Reports from analysis of volatiles in animal models showed that breath gas chromatograms from mice infected with *P. aeruginosa* is distinct than that of *S. aureus*. However, when either of these two pathogens is grown in vitro, the resulting gas chromatogram matches less than one-third of the in vivo gas chromatogram (Zhu et al. 2013). Indeed, the most challenging part in assessing volatile fingerprint from isolated microbial cultures lie in how it mimics the actual fingerprint when grown in situ.

Considering the wealth of information on microbial volatiles metabolism and advancement in detection, these obstacles should be lessened now and with more focus towards identifying volatiles using in vivo based assays and not simply by growing microbes using in vitro lab cultures. In case of *S. aureus* infection in mice (Zhu et al. 2013), observation has been confirmed in patients with ventilator-associated pneumonia. Breath VOCs analysis of *S. aureus*-infected patients matched approximately one-third of the in vitro VOCs of *S. aureus*. Moreover, exhaled breath concentration of specific VOCs was correlated with microbiological test for the presence of *S. aureus*, but faster and in a rather non-invasive way (Filipiak et al. 2015).

A. baumannii is a multidrug-resistant Gram-negative pathogen that is a common causative of ventilator-associated pneumonia and associated with increased mortality (Falagas and Rafailidis 2007). In vivo breath analysis of patients infected with *A. baumannii* exhibited characteristic VOCs profile that included: 1-undecene, nonanal, decanal, 2,6,10-trimethyl-dodecane, 5-methyl-5-propyl-nonane, longifolene, tetradecane, and 2-butyl-1-octanol. This VOCs profile was presented as a promising proxy for the presence of *A. baumannii* in patients with ventilator-associated pneumonia. Unfortunately, in vitro VOCs analysis of *A. baumannii* culture matched only 50% of the in vivo VOCs. Such discrepancies warrant for considering culture condition, metabolic niches, and host factors when relying on in vitro experiments (Gao et al. 2016). In vivo comparative analysis of rabbits with ventilator-associated pneumonia developed by either *E. coli* or *P. aeruginosa* revealed specific VOCs that could be used to distinguish between healthy and infected rabbits, as well as to differentiate between rabbits infected with *E. coli* or *P. aeruginosa* (Kunze-Szikszay et al. 2019).

Breath VOCs analysis has even further proved of potential in differentiating among different strains and not just at the species level. This was recently proved using in vivo animal model, in which mice lungs were inoculated with different strains of *P. aeruginosa* (PAO1, PA14, PAK, or PA7). Nine volatiles were identified that are able to discriminate between infected and control mice, which include isoborneol, *p*-cymene, and 2-hexanone. Furthermore, Purcaro and colleagues identified 10 volatiles in breath of mice infected with *P. aeruginosa* that could be used to distinguish different strains of *P. aeruginosa* (Purcaro et al. 2019). Among these volatiles were cyclohexanol, (*E*)-2-methyl-2-butenal, 4-cyclopentene-1,3-dione, 1,3-dimethylcyclopentanol, and 2-hydroxyethyl acetate.

From these volatiles, 2-hexanone was previously identified as *P. aeruginosa* emitted volatiles from patients sputum (Goeminne et al. 2012). Whether those volatiles contribute to the virulence of the pathogen is another area of research that is worth discovering.

As cystic fibrosis (CF) patients are a susceptible population to different pathogens causing lung infections, identifying the causative agent using non-invasive techniques is highly useful and is thus warranted. Analysis of breath from CF patients with different lung infections for their VOCs was proved useful if used as a fingerprint not to identify specific biomarkers for infectious agents (Kramer et al. 2015). One of the consistent volatile signals to *P. aeruginosa* is hydrogen cyanide.

This volatile has been detected from both in vitro and in vivo studies. Mouth- or nose-exhaled breath of adults and children CF patients infected with *P. aeruginosa* showed the presence of hydrogen cyanide. Hydrogen cyanide measurement in CF patients breath is a valuable additional diagnostic tool to detect the presence of *P. aeruginosa* in the airways. This can also serve as a non-invasive approach for the prognosis of *P. aeruginosa* lung infections. The direct real time breath analysis has been facilitated by the newly developed technique, selected ion flow tube mass spectrometry (SIFT-MS) (Smith and Spanel 2005; Spaněl and Smith 2011; Smith et al. 2013).

It should be stated that one of the co-founding factors that may affect the reproducibility and accuracy of breath analysis is diet type. Based on animals models, food intake can significantly contribute to the breath VOCs and some of these food-derived VOCs can persist in breath VOCs for hours (Fischer et al. 2015).

4.2.2.5 Oral Hygiene

Malodorous breath, also known as "bad breath" or halitosis, is a recurrent problem in 50% of the adults, in which they have a strong unpleasant breath odor that can significantly interfere with their social life (Kapoor et al. 2016). Headspace solid-phase microextraction-gas chromatography/mass spectrometry (SPME-GC/MS) was used to assess volatiles in malodorous breath and saliva to better understand oral hygiene. Numerous VOCs were detected such as sulfur compounds, phenol, and indole. Sulfur compounds are produced mainly by proteolytic bacteria (Rösing et al. 2002). Phenol and indole are bacterial fermentation by-products of aromatic amino acids and tryptophan; thus, such studies need to be complemented with oral microbiome research to better understand the microbial community function in oral malodor (Payne et al. 2000).

Research attempt to correlate the severity of oral malodor with saliva microbiome found an association between lower oral malodor and higher proportion of these genera *Streptococcus*, *Granulicatella*, *Rothia*, and *Treponema* in the saliva microbiome. More importantly, severe oral malodor was found to be associated with different saliva bacterial composition rather than higher bacterial count (Takeshita et al. 2010).

Porphyromonas gingivalis is another pathogenic Gram-negative anaerobic bacterium that can invade periodontal tissues and contribute to oral malodor due to its volatile sulfur compounds, e.g., 3-bromo-5-chloro-1-tosyl-1*H*-pyrrolo[2,3-*b*]pyridine. In vivo and in vitro studies showed that egg yolk immunoglobulin can inhibit the growth of such pathogen and protect against periodontal inflammation and oral malodor (Qiao et al. 2018).

Dental caries is a frequent disease worldwide that has been linked to different oral bacterial species. Thus, the development of a simple and reliable caries risk assessment based on microbiome VOCs seems of value. In vitro VOCs analysis of potential cariogenic species such as *Streptococcus mutans*, *Lactobacillus salivarius*, and *Propionibacterium acidifaciens* revealed some unique volatiles indicative of

each species, however, in vivo analysis is critical to confirm whether these volatiles are biologically relevant (Hertel et al. 2016).

4.2.2.6 Skin Hygiene

Axillary malodor has been a longstanding cosmetic issue. To understand its origin, it was pivotal to characterize the axillary sweat composition, skin microbiome, and the malodor volatiles. Artificial sweat composition was created and incubated with skin microbiome. The in vitro malodor volatiles showed correlation with the in vivo axillary malodor components. These compounds comprised volatile acids (acetic acid, propanoic acid, isovaleric acid, butanoic acid, myristic acid, pentadecanoic acid, and palmitic acid), ketones (2-butanone, 2-pentanone, 2-nonanone, and 2-undecanone), aldehydes (2-pentanal, 1-hexanol, octanal, and nonanal), and *n*-hexadecanoic ester (Callewaert et al. 2014). This is a promising area of research to understand the role of different bacterial species in the production of axillary malodor.

4.2.2.7 Tuberculosis

Tuberculosis is a global threat to humanity and is the second most common cause of death from infectious diseases. It is caused by *Mycobacterium tuberculosis,* with humans are its only reservoir (Khatua et al. 2017). Considering that *Mycobacterium* species are causative agents in pulmonary infections and have high antibiotic resistance and mortality rate, an increasing interest has been given towards diagnosing pulmonary tuberculosis using VOCs in breath. Numerous studies were conducted in vivo and in vitro to identify VOCs that are associated with *Mycobacterium* pathogenesis and assess their potential role as potential diagnostic biomarkers (Phillips et al. 2007; Syhre and Chambers 2008; McNerney et al. 2012; Bergmann et al. 2015; Mellors et al. 2018). Unfortunately, in vivo and in vitro results were irreproducible and found more dependent on either the animal model or the adopted culturing approach (Dang et al. 2013). Furthermore, specificity of VOCs is another limiting factor that need to be seriously considered in experimental design and data analysis. For example, one of the few volatiles that was found consistently and presented as *M. tuberculosis* breath biomarker is heptanal. However, it is non-specific as it was also considered as a maker in other lung cancer patients (Pereira et al. 2015; Mellors et al. 2018). Considering the complex matrix of volatiles normally released from human breath, specificity of certain microbial VOCs is an issue that need to be critically determined for posing it as marker for certain lung disease incidence.

4.2.2.8 Wound Infections

Biofilms are a common life forms for several bacterial species, in which bacterial cells attach to a surface while incasing themselves within an extracellular polymeric substance matrix, in comparison to planktonic cells that float as single cells in the environment (Donlan 2002). Recently, the prevalence of biofilms in chronic wounds was studied for their contribution in delaying wound healing, and consequently pursued to be more characterized using different approaches (Attinger and Wolcott 2012). Ashrafi and colleagues characterized VOCs of planktonic and biofilms in vitro cell culture model for *S. aureus*, *P. aeruginosa*, and *S. pyogenes* (Ashrafi et al. 2018). *P. aeruginosa* biofilms rather than planktonic cultures emitted unique VOCs such as hydrogen cyanide, 5-methyl-2-hexanamine, 5-methyl-2-heptanamine, 2-nonanone, and 2-undecanone. In contrast, *S. aureus* and *S. pyogenes* did not show difference in their planktonic and biofilm VOCs profile. To take that model a step further, Ashrafi and colleagues compared VOCs of the same pathogens when grown using an ex vivo model of biofilms formed on cutaneous wounds. Such an ex vivo model characterized the specific VOCs profile of clinically important pathogens, *S. aureus*, *P. aeruginosa*, and *S. pyogenes,* that could be further exploited to non-invasively diagnosis of the different wound infections.

4.3 Challenges in In Vivo Detection of Bacterial Volatiles Compared to In Vitro Methods

Even with the most recent advances of analytical methods to analyze VOCs in vitro, it was clear that numerous challenges still exist to adapt these methods to the in vivo situations and/or studies. For example, in a clinical laboratory for disease diagnosis, the required instruments for VOCs analysis are rather expensive, relatively bulky, and need specialized training and expertise to analyze samples and resulting data. Moreover, depending on the employed technique, some require sample preparation and lengthy time for analysis that are not always scalable for high-throughput screening.

The concept of finding a single volatile biomarker has shown failure over the years, due to the non-specificity of such VOCs, the irreproducibility of such results, lack of procedure standardization, and proper controls. Therefore, a growing interest in identifying whole VOC profiles, which may comprise hundreds of VOCs, has been documented and shown clear potential (Nizio et al. 2016). However, this approach also suffers from certain limitations. For example, VOCs production is environment-dependent, and the VOCs profile is at least 30% different when the same pathogen is grown in different culture media. Also, other factors such as the bacterial growth phase and sample storage conditions can affect the detected VOCs profile (Tait et al. 2014; Nizio et al. 2016). These numerous factors that may influence the VOCs profile by bacteria are summarized in Fig. 4.2. Another concern

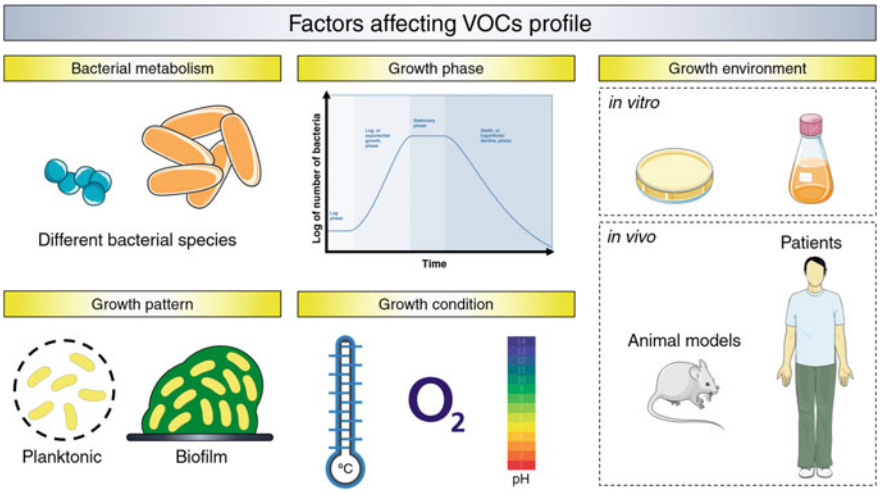

Fig. 4.2 A summary of different factors that may affect the production of VOCs by bacteria. These factors comprise the bacterial metabolic pathways that are distinct among different bacterial taxa as a consequence of different genetics, sometimes at the strain level. Other factors include growth phase of the bacterial species, whether cells are grown as planktonic or in a biofilm, environmental factors such as temperature, oxygen concentration, and pH, lastly, growth environment. VOCs profile was found to be around 30% only similar between in vivo and in vitro studies. Animals models have also been used to study VOCs in different infections and show some discrepancy than human infections

was raised up owing to the biological variability among the same patient population; thus, VOC profiles identified in a few number of patients as a potential diagnostic fingerprint require careful consideration before it can be generalized (Fend et al. 2006; Hong-Geller and Adikari 2018).

Finally, as researchers are attempting to identify unique VOCs produced by pathogens, it is imperative not to underestimate that the human body lives within consortia of microorganisms including bacteria, and it is quite difficult to find that unique volatile to a pathogen that is not produced by the human microbiome.

4.4 Conclusions

This chapter reports on the potential role of many microbial VOCs as airborne signals or environmental cues in agriculture and medicine. Over the past few decades, numerous research groups have developed various technologies to analyze and use VOCs to diagnose pathogenic infections. Biomarkers have been identified to discriminate between a handful of pathogens using in different in vivo and in vitro models. VOC profile fingerprints have also been established to distinguish between different pathogens and proposed to be more valuable, even without the need to

identify single volatiles. As previously discussed, further developments of those techniques are still needed to have point-of-care testing tools to analyze VOCs in vivo that are sensitive, fast, and cheap. In the near future, it is possible with deeper VOC analyses of different pathogenic species in possible infections that we will be able to establish strong reliable volatile-specific sensors for the differential diagnosis of complex diseases. The use of point of care devices has never been reported for microbial VOC detection in diagnosis and we foresee it as potential approach towards applying fabricated simple devices for disease diagnosis via its volatile fingerprint. In a broader perspective, the production, regulation, activity, and sensing of VOC in recipient microbes are still inadequately characterized. Furthermore, combining metabolomic and transcriptomic analyses will be a powerful approach to identify candidate genes involved in the production of biologically active volatiles.

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Chapter 5
How Plants Might Recognize Rhizospheric Bacterial Volatiles

Aurélien Bailly

Abstract In contrast to animals, plants possess neither olfactory organs nor a central nervous system. However, they do perceive and systemically react to volatile stimuli. Such function serves in monitoring the immediate and remote environments and translates into optimized responses to biotic and abiotic stresses. While the ecological relevance of volatile-mediated plant–plant and plant–insect interactions is today unquestioned, both above- and below-ground plant–microbe communication through VOCs has only gained attention recently. The common metabolic origins that yield the vast chemical diversity of plant and microbes allow for a substantial overlap between plant and microbial volatile species. Hence, it remains unclear if plants recognize and/or distinguish plant-like from foreign cues. The identities of the cellular components ensuring such recognition are even more obscure. Easy-to-score plant outputs in response to microbial VOCs elicitation, like plant growth promotion and innate immunity stimulation, will be instrumental to pinpointing VOCs-sensing proteins. Several major phytohormones have a gaseous nature and dedicated perception machineries that could serve as a basis to envisage how volatile semiochemicals might be sensed by plants. If volatile-mediated communication represents an ancestral cellular feature, VOCs perception and signalling might rely on basal protein families and define a universal chemical language.

Keywords Volatile organic compounds · Semiochemical · Perception · Receptor · Transduction · Self · Non-self

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5.1 Introduction

In the middle of the twentieth century, a Science article entitled "The *raison d'être* of secondary plant substances", by German entomologist Gottfried Fraenkel, set the foundations of plant chemical ecology (Fraenkel 1959). Beyond identifying a central role of secondary metabolites produced by plants in their interactions with insects, it put forward an evolutionary link between the selective pressures imposed by herbivory and plant chemical diversity.

In the great majority of ecosystems, the constant competition between the plant kingdom, primary source of complex organic compounds, and heterotrophs has driven an arms race between plants and their enemies. Per definition, secondary metabolites do not enter into the basal metabolism required for the plant growth and reproduction cycle, and therefore represent a pool of substances that can eventually be released to the extracellular environment at lowest cost. The genetic machinery leading to the production of such chemicals has certainly been prone to permissive mutations during evolution, thus granting the plant kingdom with a considerable level of chemical diversification and sophistication (Jenke-Kodama 2008; Moore et al. 2014; Maeda 2019).

This chemical repertoire is nowadays considered key to the communication strategies that plants, sessile in nature, build with their biotic environment. While the ability of plants to convey and perceive chemoinformation is not anymore debated, the extant of such aptitude is far from being well comprehended. Mutualistic behaviours between plants and pollinators, mycorrhiza, nitrogen-fixing bacteria or other plants have been extensively documented, down to the molecular level, hence increasingly describing the flora as a realm of chemical languages. This chemical arsenal serves as well in the plant defence systems, either directly or indirectly. Plant behaviours consisting of deterring or repelling pests via specific compounds have been continuously reported in the past decades (Heil 2014; Noman et al. 2020) and chemical attraction of natural enemies of herbivore insects are common strategies described in land plants (Kessler and Baldwin 2001).

The large majority of these ecological interactions rely on biogenic, low molecular-weight volatile organic compounds (VOCs) and, as marine and land plants are the major atmospheric VOCs contributors (Lerdau et al. 1997), their aptitude to emit airborne semiochemicals is obvious. However, after a long period of scepticism, it is only recently that the scientific community reached a consensus about plant VOCs perception. First reports from the 1980s revealed that undamaged plants nearby conspecifics under wounding or herbivory stresses altered their biochemical status, thus becoming less prone to infestation by grazers compared to distant individuals (Baldwin and Schultz 1983; Rhoades 1983). Such pioneer results strongly suggested that plant–plant chemical communication can occur in the shared headspace of individuals and may represent a social behaviour. This discovery consequently raised unsought questions that opened a completely new area of plant research: are VOCs-based communications hereditary, specific signalling events? Do plant volatile conversations only involve kins or address to broader

communities? What chemical species participate in these signalling mechanisms? How do plants perceive such signals? After several decades of work, the scientific community has tremendously progressed in our understanding of these phenomena and a number of recent articles will guide the reader through this fascinating field or research (Baldwin et al. 2006; Heil and Karban 2010; Heil 2014).

Animals constantly survey and recognize chemical cues from the environment as they carry stimuli indispensable for achieving optimal behaviours. Olfaction is probably the most prominent and specific sensory apparatus in invertebrates and an essential function in vertebrates. Evolution granted these groups with dedicated molecular machineries that ensure the identification of VOCs and relay the information to decision centres. Surrounded by the same environments and being incapable of avoiding local stresses, it seems reasonable to assume that plants would take advantage of a rapid and/or early detection of volatile semiochemicals to adapt their physiology to upcoming threats. Beyond sensing danger, such compounds could simply serve to directly monitor and evaluate the immediate environment.

Far from being restricted to eukaryotes and large organisms, VOCs production also occurs in microbes where they most probably serve similar informative and defence functions (Schulz et al. 2020; Lemfack et al. 2020). It is common knowledge that bacteria and fungi emit pungent smells that can, for example, rapidly attract flies to cattle dungs or prevent animal feeding on fouled food sources. Plants do form an intricate ecological web with microorganisms; therefore, it seems plausible that sensing and assessing these microbiota via their volatilomes could represent a valuable input for tuning plant growth and defence responses. Surprisingly, it is only in recent years that this hypothesis was translated into experimental evidence (Ryu et al. 2003, 2004; Paré et al. 2005; Farag et al. 2006; Yi et al. 2009). After the initial in vitro assays that described the effects of microbial volatile organic compounds (mVOCs) on plant growth behaviour and immune response elicitation, similar results were reported in several plant–microbe systems and tentatively implemented into experimental setups closer to natural settings (Brilli et al. 2019), thus implying that plant perception of mVOCs is a widespread mechanism. However, despite more of a decade of research, the means by which plant cells detect microbial volatile scents remain obscure.

This chapter will specifically address how plant species may perceive such odours. The sum of literature available to date clearly delineates the role of volatile cues in ecological contexts, and plant responses to such molecules have been relatively easy to monitor and interpret in well-investigated genetic frameworks such as immunity and growth. Despite mounting evidence that various other plant reactions, including abiotic stress responses, are also impacted by VOC signals, the involved molecular components and circuitries bridging volatile signals and downstream physiological alterations have been less investigated. Thus, parallels between plant-borne volatile communication and a presumed environmental mVOCs perception can be drawn without difficulty when one focuses on plant–microbe interactions.

5.2 The Plant Immune System Integrates Volatile Chemical Cues

From their onset of life, land plants are virtually covered by environmental microorganisms colonizing their surfaces, invading intra- and intercellular spaces or building intimate symbioses. Microbes have evolved different life strategies displaying commensal, beneficial or pathogenic behaviours towards plants to gain access to the profuse metabolic resources they offer. Sessile plant belowground and aerial organs are therefore constantly challenged with biotic cues that need to be processed in order to balance growth, development and defence programs and reach optimal fitness. Devoid of a central nervous system, a somatic adaptive immunity and mobile defender cells, plants developed a multi-layered monitoring strategy that relies on the capacity of each individual cell to perceive molecular effectors. Upon perception, such cues are integrated, sorted and translated into a systemic signal that triggers an alert status in distant organs and on-site defence responses. This well-documented innate immunity interprets different effector classes into dedicated responses: (1) relatively conserved microbial- or pathogen-associated molecular patterns (M/PAMPs), such as flagellin, lipopolysaccharides or fungal chitin, are first detected at the plant cells surface by transmembrane pattern recognition receptors (PRRs) and activate PAMP-triggered immunity (PTI); (2) effector molecules delivered inside host cells, as through the bacterial type III secretion system, can suppress basal plant defence responses and favour the establishment of diseases, or be recognized by NB-LRR (nucleotide-binding leucine-rich repeat) domain proteins that initiate effector-triggered immunity (ETI) (Jones and Dangl 2006; Boller and Felix 2009). After successful recognition, the confinement of the potential invader is ultimately achieved through the local apoptotic hypersensitive response (HR) and the reinforcement of cell wall barriers.

PTI and ETI events trigger the systemic acquired resistance (SAR) in plant tissues remote from the infection site, thus granting uninfected plant parts the ability to oppose, at the next encounter, an enhanced defensive response against a broad spectrum of pathogens (Boller and Felix 2009; Henry et al. 2012).

Although this conceptual approach of plant–microbe interactions, mainly based on pathogen effector recognition, is of fundamental importance for plant life, it represents only the tip of the iceberg. The vast majority of plant-associated microorganisms do not cause disease; still, these species present molecular motifs to the plant similar in nature to pathogenic ones. In the beginning of the 1990s, beneficial rhizobacteria known to contribute to and promote plant growth and health were shown to trigger an induced systemic resistance (ISR), greatly relying on the defence-related plant hormones jasmonic acid (JA) and ethylene (ET) signalling pathways and distinct from SAR that confers to the plant protection against a large array of microbial threats (Doornbos et al. 2011; Pieterse et al. 1998, 2014). It is worth noting that the independence of ISR towards SAR is currently under debate and both responses can be envisaged as different branches of a common defence program. This is supported by the fact that both pathways converge to the

transcriptional co-regulator, SA-receptor NPR1 (NONEXPRESSOR OF PR GENES1), demonstrated to integrate SA- and ET/JA-dependent immunity signals via its nuclear and cytosolic compartmentalization, respectively (Kinkema et al. 2000; Spoel et al. 2003; Dong 2004; Pieterse and Van Loon 2004; Zhang et al. 2010; Van der Does et al. 2013).

The plant ability to survey environmental threats is not limited to microbial effector perception. SAR can be also triggered upon wounding, herbivory or tissue damages such as those caused by intruding aggressors. In contrast to PTI and ETI, plants recognize their endogenous, plant-derived signals released during the injury called damage-associated molecular patterns (DAMPS) (Boller and Felix 2009; Henry et al. 2012). In analogy, plants release a number of volatiles upon wounding or microbial recognition, including ET, terpenoids and phenylpropanoids, as well as lipoxygenase-derived compounds such as C6-volatiles (C6V or green leaf volatiles, GLVs) and methyl jasmonate (MeJA). These semiochemicals are rapidly produced and/or emitted at, or distal to the attack site, can travel through the air and water phases and eventually also elicit plant defences (Turlings and Tumlinson 1992; Engelberth et al. 2004; Heil and Bueno 2007; Heil 2010; War et al. 2011). These responses have been mostly reported in their ecological context, especially during herbivores grazing, and data demonstrating the molecular mechanisms underlying particular compounds' perception are scarce. For example, the monoterpenes myrcene and ocimene were shown to elicit *Arabidopsis thaliana* defence responses close to those triggered by mechanical wounding, insect feeding or MeJA treatments (Engelberth et al. 2004). Nevertheless, one tentative concept emerges from the current literature: on the one hand, plants have the ability to recognize and respond to defence metabolites released from either their own selves, conspecifics or from emitters of different species; on the other hand, the large majority of these molecules are rather simple, secondary compounds that can virtually be produced by many organisms. As our detection capacities progressed, mainly through gas chromatography-mass spectrometry (GC/MS) methods, microbes, and particularly bacteria, have been increasingly reported to generate an outstanding chemical diversity in volatiles production (Kanchiswamy et al. 2015).

5.3 Microbial Volatile Organic Compounds Stimulate Growth and Prime Systemic Resistance in Plants

Therefore, in the light of these assumptions, the relatively recent discovery that, under laboratory setups, rhizospheric bacteria and fungi prime systemic resistance in plants via their biogenic volatile organic compounds is of utmost interest (Ryu et al. 2005; Bailly and Weisskopf 2012; Bitas et al. 2013; Peñuelas et al. 2014). Indeed, model plants such as *A. thaliana* challenged in vitro with complex VOC blends from physically separated microorganisms were shown to activate molecular defence pathways unequivocally resembling ISR and/or SAR (Bitas et al. 2013). This finding

originally focused on plant-growth promoting rhizobacteria and fungi (PGPR/F), for the ability of their microbial scents to drastically enhance plant biomass (Ryu et al. 2003; Bailly and Weisskopf 2012), but no available data exclude the possibility that pathogenic strains, devoid of poisonous volatile species such as hydrogen cyanide or ammonia, hold the same properties. When compared to PTI, ETI or SAR, the recognition of VOCs and subsequent triggering of systemic defence seems rather unspecific since VOCs challenges were reported to confer resistance to pathogens unrelated to the VOC emitters (Bitas et al. 2013; Pieterse et al. 2014; Venturi and Keel 2016). Taking into account that microbial colonizers form dynamic communities on the plant surface, it is henceforth very tempting to question how plants deal with the interpretation of this confusing sum of signals. Undeniably, the rhizosphere represents an environment conductive to dynamic and complex interspecies exchanges where the release and perception of volatile cues could be an asset for the establishment of sustainable life strategies. Therefore, a fine-tuned regulation of the plant immune response is essential to balance other physiological processes, such as growth and development, as the synthesis of defence-related products has a high metabolic cost (Van Hulten et al. 2006).

5.4 Balancing Immune Response and Fitness Costs

Given the fact that not only PGPR volatiles but also direct contacts with the plant host can promote plant growth and modulate root architecture, it seems reasonable that other phytohormones outside of directly defence-related ones play critical roles during plant–microbe interactions (Bailly and Weisskopf 2012; Denancé et al. 2013). The role of auxins, the most prominent coordinators of plant organogenesis and growth, has not been exhaustively studied in this context. Nevertheless, many components of the auxin signalling pathway were shown to be regulated during the immune response, including receptors, transporters, transcription factors and biosynthetic and conjugating enzymes (Fu and Wang 2011; Denancé et al. 2013). In addition, elevated auxin contents antagonize resistance against microbial invaders and several of them mimicry or manipulate auxin homeostasis to enhance their virulence (Fu and Wang 2011; Denancé et al. 2013). Direct crosstalks between auxin-, SA- and ET-signalling pathways are also to be taken into account, and many auxin mutants are impaired in systemic immunity (Truman et al. 2010). The native function of auxin itself disserves the defence program as auxin-mediated cell elongation weakens cell walls and counteracts the reinforcement of natural barriers. Finally, the obvious pivotal role of indolic precursors leading to either auxin biosynthesis or defensive tryptophan-derived phytoalexins and indole glucosinolates set auxins at the crossroads of the VOCs-triggered plant growth promotion and immune response. Although we will not specifically address the VOC-mediated plant growth promotion here, the overlap of development and defence signalling routes should be kept in mind (Kim and Anderson 2020). The reported growth

effects triggered by VOCs could be a consequence of an altered immune status and vice versa.

5.5 Volatile Organic Compounds May Represent an Ancient, Universal Communication Strategy

It is established that VOCs emissions generally intensify when plants are endangered (Unsicker et al. 2009; Holopainen and Gershenzon 2010). However, all plant species constitutively emit volatile compounds, virtually from all organs (Knudsen et al. 2006; Pichersky et al. 2006). As they essentially derive from primary as well as secondary metabolic pathways, the transcriptional regulation of their production is poorly understood (Dudareva et al. 2013), and the identity of volatile metabolites produced by plant species is extremely difficult to predict solely from genomic information. Hence, the identification of these compounds relies on detection methods and study conditions (Blom et al. 2011). Although the library of known higher plants' VOCs now exceeds 2000 chemicals, one can foresee a continuous increase of this number in the years to come. Despite the current ease of volatiles collection and the interest in understanding their influence on plant communities, the chemical identification of bioactive species remains a limiting step (Vivaldo et al. 2017). An improvement of our knowledge in the VOCs emitted by plant below ground organs is especially sought after, for that the rhizosphere hosts rich and complex communities (Delory et al. 2016; Gfeller et al. 2019; Huang et al. 2019). This remarkable chemical diversity anyhow contrasts greatly with the limited number of biosynthetic pathways known to lead to VOC production in plants. Dudareva and colleagues classified most of these chemical species in four major classes, depending on their primary metabolic origin: (1) terpenes, (2) phenylpropanoids, (3) fatty acid derivatives and (4) amino acid derivatives; yet, the source of plant VOCs is not restricted to these primary building blocks (Sun et al. 2016). Moreover, these basic metabolic routes are far to be exclusive to the plant kingdom and the diversity of volatiles from microbial origin is probably alike to the plant volatilome, if not greater (Schulz and Dickschat 2007; Hung et al. 2015; Kanchiswamy et al. 2015; Schmidt et al. 2015; Lemfack et al. 2017, 2020; Schulz et al. 2020). In contrast to fungi, bacteria do not synthetize phenylpropanoids, but they produce a broad range of benzenoids and several species possess biosynthetic toolkits close to plant ones (Moore et al. 2002). Terpene synthetases were recently identified in bacterial genomes (Yamada et al. 2015a) and terpenoids are common fungal products. Many microbial VOCs derive as well from lipids and amino acids. It is therefore tempting to assume that a non-negligible overlap between plant and microbial volatile chemical mixtures occurs (Fig. 5.1a). Such approach resulted in the identification of 28 volatile species shared between plant root and microbial VOCs (Schenkel et al. 2015). Although limited to 39 root compounds identified from four plant species compared to >1000 microbial VOCs, the data reported by Schenkel and co-workers

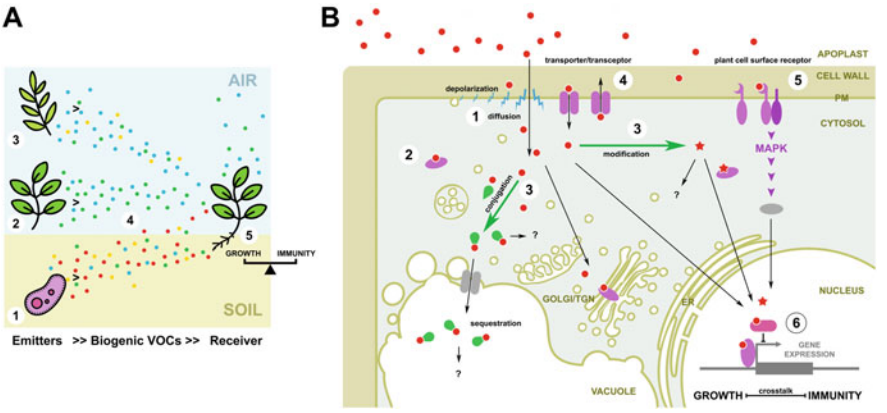

Fig. 5.1 (**a**) Biogenic volatile organic chemicals (VOCs) can carry information to plants over relatively long distances. Microorganisms such as bacteria (*1*), conspecific (*2*) and heterospecific (*3*) plants release a large variety of VOCs into the surrounding environment. These semiochemicals can be either particular or common to the emitters (*4*). Recipient plants respond to these stimuli, therefore integrate the chemical signals through below- or above-ground organs (*5*). (**b**) Individual plant cells might perceive the volatile cues (red dots) through different mechanisms. (*1*) VOCs physico-chemical properties allow passive diffusion of the compounds through the extracellular matrix and biological membranes, hence virtually targeting all subcellular compartments. Alteration of transmembrane potentials caused by VOCs might lead to downstream signalling. (*2*) Cytosolic, as well as organelle-localized enzymes or receptor-like proteins might directly sense and trigger cellular responses to VOC thresholds. (*3*) VOC species could undergo enzymatic alteration or conjugation to yield soluble active forms or be isolated to subcellular compartments. (*4*) Transceptor-like integral membrane proteins might convey VOC information by (selectively) importing or exporting them across cellular membranes. (*5*) Cell-surface receptors might sense volatile cues and transduce signals via phosphorelay cascades. (*6*) VOCs might directly target nuclear components controlling gene expression, such as transcription factors or repressors

let space to a scenario where microbes indeed produce VOCs identical or structurally close to plant ones, as do bacteria and fungi which share several volatile species. These results also demonstrated that a number of VOCs might be specific to plants, bacteria or fungi. Consequently, in view of the mounting importance given to VOCs in belowground chemical interactions between soil inhabitants (Schulz-Bohm and Martín-sánchez 2017), it is sound to interrogate ourselves on the plant capacities to disentangle the multifarious volatile signals occurring in the rhizosphere. If plants only possess dedicated systems to perceive plant-borne VOCs, one would expect that the exact same ligands triggering these systems, but emitted by microbes, would certainly lead to similar responses in the recipient individuals. Such an organization would greatly limit the plant perception of the rhizosphere to an array of predefined signals that are not representative of the soil biodiversity. Moreover, these semiochemicals would then carry confusing information. Contrariwise, if plants evolved a supplemental layer of molecular machineries to detect foreign volatile molecules, it seems rather implausible (but not impossible) that a limited number of plant protein families could ensure the specific recognition of the countless VOCs

unambiguously produced by microorganisms. Furthermore, a model in which plants would inform themselves on potential harmful microorganisms through VOCs is counterintuitive. Indeed, nothing indicates that the ground metabolic source of a vast majority of sampled microbial volatiles might yield different molecules in pathogenic or non-pathogenic strains of similar species. As the pathogenicity of different, but closely related microbial strains (e.g. pathovars) relies on highly specific effector proteins undoubtedly disconnected from volatile emissions, such a model is not satisfying. It was demonstrated that VOCs emitted by phylogenetically distinct microbes outside beneficial rhizospheric bacteria and fungi also promote plant growth and the transcriptome changes in plants exposed to volatiles of the fungal pathogen *Alternaria alternata* resembled those of plants subjected to VOCs emitted by the PGPR *Bacillus subtilis* (Sánchez-López et al. 2016). Additionally, a study comparing the impact of pathogenic and non-pathogenic soil fungi volatiles on Arabidopsis phenotypic responses suggested that the plant does not distinguish between friends or foes via their VOCs signatures (Moisan et al. 2019). Although the authors claimed the volatilomes of non-pathogenic and pathogenic fungi distinct, the study mostly compared the chemical headspaces of discrete fungal species. A more direct comparison of two strains sampled in this analysis, *Fusarium oxysporum* f. sp. *raphani* and *F. oxysporum* Fo47, showed that their volatilomes indeed differ, but they did not produce any of the of 11 chemical species identified as the main contributors to the separation between pathogenic and non-pathogenic fungi volatilomes. The phylogenetic distance between these *F. oxysporum* strains could explain these discrepancies, as it was shown that very closely related Pseudomonas isolates produce similar but distinct chemoprofiles (De Vrieze et al. 2015; Bailly and Weisskopf 2017). Blom et al. (2011) clearly evidenced that the composition of volatilomes is directly dependent on feeding media, thus suggesting that marker compounds produced in one metabolic situation may not be representative of another, and that VOC signals are susceptible to vary in intensity upon resources availability. The subtle equilibrium of volatile chemoprofiles is deeper illustrated by the strong variations in VOC emissions observed between Pseudomonas isogenic mutants and their respective wild types (Bailly et al. 2014; De Vrieze et al. 2015; Bailly and Weisskopf 2017). Therefore, it seems unlikely that plants rely on diverse, changing cues to mount precise responses against invaders. This is probably reflected in the priming effects on innate immunity and the broad-range resistance conferred to plants by VOCs against pathogens. Even if one cannot exclude that particular pathogenic microorganisms might use volatile chemical tools for successful invasion, such as virulence factors, and that particular plant species under selective pressure invented means to evade their enemies, a general survey strategy that provides plants with the ability to monitor and assess the belowground microbial community appears like a secure tactic choice at low metabolic cost. As stated previously, the profuse volatile cues produced by different organisms within the rhizosphere, including by plant roots (Venturi and Keel 2016; Delory et al. 2016), did not yet allow a comprehensive dataset of VOC species emitted by individual microbes in situ. The lack of qualitative and quantitative information of the volatile

compounds produced by microbes when feeding on the root surface or in the rhizosphere currently represents an enormous knowledge gap.

To add up to the complexity of finding how plants perceive and recognize volatiles, a large body of literature suggests that naturally occurring blends of VOCs, and probably their spatio-temporal distribution, are more effective in stimulating plant responses than single molecules (Ueda et al. 2012; Šimpraga et al. 2016).

To date, no *bona fide* receptor has been unambiguously demonstrated to participate into the binding of non-plant VOCs, and the information available on the mode of sensing of plant VOCs remain limited. However, plants greatly rely on volatile species for crucial biological functions that received early attention. Indeed, several phytohormones belong to VOCs and their modes of transmission, from cell to cell, organ to organ or plant to plant have been extensively studied and could serve as a solid basis to predict which protein families partake into VOC perception (Fig. 5.1b).

5.6 Hormonal VOCs

Ethylene is undoubtedly the simplest and earliest volatile signal to have been described essential to plant development. Ethylene is biosynthesized from methionine during development and upon stress stimuli comprising herbivory and wounding. As the vast majority of VOCs, ET is small, apolar and can readily diffuse across cell membranes. Past the plasmalemma, ET binds to a family of structurally distinct receptors on the endoplasmic reticulum (Wang et al. 2013). EIN1/ETR1 was first characterized in a forward genetics screen in Arabidopsis for ET insensitivity (Chang et al. 1993). In the following years, four additional ethylene receptors with overlapping roles (ETR2, EIN4, ERS1 and ERS2) were identified (Hua et al. 1995, 1998; Sakai et al. 1998), thus demonstrating the critical importance of ET signalling. ET-receptors are histidine-kinases that display homology to prokaryotic two-component receptors, a widespread signal transduction system enabling the perception of an extensive array of stimuli, from quorum sensing molecules to chemoattractants and pH sensors. Next, the ET binding events prevent the activation of a Raf-like (MAPKKK) protein kinase (CTR1 in Arabidopsis (Huang et al. 2003) and downstream phosphorelay leads to transcription factors activation (ERF, EIN) followed by the regulation of ET-dependent gene expression). Ethylene perception thus represents an intuitive model for the volatile-mediated relay of information coming from neighbouring cells or remote sites. One could easily imagine that most VOCs potentially holding a precise, evolutionary conserved message would favour this classical mode of transmission. However, in contrast to the large number of receptors found in prokaryotes, the number of two-component system receptors in plants is restricted to *ca.* 20 proteins [e.g. 16 in Arabidopsis (Hwang et al. 2002), 25 in banana (Dhar et al. 2019) and 18 in chick pea (Ahmad et al. 2019)] mostly dedicated to hormonal and light signalling. Although the full extent of plant histidine-kinase phosphorelay circuitry is not entirely decrypted, the probability that such system convey supplementary specific volatile signals is low.

A second example of a VOC-type phytohormone is methyl salicylate (MeSA), the methyl ester of salicylic acid (SA). SA is well known for its pivotal role in coordinating the systemic acquired resistance (SAR) plant defence strategy against pathogens (Kumar 2014). SA is a simple benzoate synthesized from phenylalanine or chorismate (Chen et al. 2009) that, under stress conditions, undergo methylation by carboxyl methyltransferase activity to give MeSA (Shah et al. 2014). The resulting volatile then serves as a cell-to-cell or remote airborne signal to trigger SAR in tissues nearby the infection site. Nonetheless, contrary to the direct processing of ET, MeSA needs to be converted back to SA by methylesterases once entered into the cytosol to show activity (Shah et al. 2014). The following steps of SA signalling remain unclear but, in Arabidopsis, a number of transcriptional co-activators/co-repressors paralogous to NPR1 have been shown to bind the hormone and control downstream SAR gene regulation (Canet et al. 2010; Castelló et al. 2018; Ding et al. 2018; Innes 2018). Beyond the precise molecular mechanisms underlying MeSA signalling, it is interesting to emphasize that the transition from MeSA to SA is a prerequisite to its effectiveness. Consequently, conceptualizing that a vast majority of VOCs can reach the plant cytosol, further chemical modifications of the original molecule are likely to occur. Plant genomes are rich in modifying enzymes that, beyond detoxification purposes (Coleman et al. 1997; Wolfe and Hoehamer 2004), regulate hormones action throughout development and stresses (Westfall et al. 2013; Xu et al. 2015). Amino acid or glutathione conjugation, glycosylation, methylation or demethylation are prominent examples of the capacity of plant cells to convert metabolites or xenobiotics in order to activate or inactivate them. Thus, the very VOCs identified in microbial emissions might not be directly sensed at the plasma membrane interface, but could accumulate in the cytosol or specific organelles like the ER or Golgi, the vacuole or peroxisomes in alternative forms. In the same extent, the original or modified plant- or microbe-derived compounds could be conveyed by signalling transporters equivalent to the NRT1.1 nitrate transceptor (Giehl and von Wirén 2015) or simply cross several lipid bilayers to act on nuclear targets.

The molecular fate of a third class of volatile phytohormones, the jasmonates (including methyl-jasmonate and (Z)-jasmone), displays a slightly different *modus operandi*. Jasmonic acid (JA) is biosynthesized from linolenic acid through the lipoxygenase pathway and is key to growth and development processes. It has also been clearly associated with damage stresses, especially wounding and herbivory. In analogy to SA, JA is converted into methyl jasmonate (MeJA) by a specific carboxyl methyltransferase, or decarboxylated to give (Z)-jasmone in response to stress stimuli. MeJA can then signal danger to neighbouring cells, travel to distal sites or inform distant plants through air. However, MeJA remains inactive in plant cells until it is first demethylated via methylesterase activity and subsequently conjugated to isoleucine. In Arabidopsis, conjugation is ensured by the GH3-family member jasmonate-amido synthetase JAR1 (Staswick et al. 1992) and yields the active form (+)-7-iso-jasmonoyl-L-isoleucine. The conjugate is then recognized by the nuclear COI1-JAZ co-receptor complex (Chini et al. 2007, 2009; Yan et al. 2009; Sheard et al. 2010) and, upon JAZ ubiquitination and degradosome targeting, frees the

transcription factor MYC2 (Lorenzo et al. 2004). The transcription factor then orchestrates the transcriptional programs by recruiting other transcription factors. (Z)-jasmone is assumed to effect gene expression through a yet uncharacterized pathway, independent of the COI1-JAZ complex.

5.7 Non-hormonal VOCs

In plants, evolution opted for simple volatile molecules to support vital functions, inferring that the physico-chemical properties of these particular organic compounds are the best fit to rapidly and precisely deliver information throughout the plant body and to remote locations. These peculiar properties are also valuable in other classes of non-hormonal semiochemicals directly involved in plant defences.

The first we will mention here is indole, an *N*-heterocyclic aromatic volatile abundantly produced in bacteria and believed to act as a *trans*-kingdom messenger (Lee and Lee 2010; Bailly et al. 2014; Erb et al. 2015). In plants, indole is the direct precursor of tryptophan and originates from the chorismate pathway. Exogenous indole was reported to prime plant defences against microbes (Shen et al. 2018), to partake into herbivory responses (Erb et al. 2015) and to promote growth (Bailly et al. 2014). The molecular events following indole entry into cells and systemic transport have not been characterized in detail, but they ultimately lead to a modulation of the SCF^{TIR1} receptor-dependent auxin signalling (Bailly et al. 2014). Auxin binding to SCF^{TIR1} promotes its interaction with the Aux/IAA transcription repressors and initiates their ubiquitin-dependent proteolytic degradation. In turn, the derepression of the auxin response factor (ARF) family of transcription factors transduces the hormone signal to gene expression regulation. At least a portion of the intracellular indole undergo a conversion to the chemically close auxinic hormone indole-3-acetic acid (IAA) (Bailly et al. 2014). It has been suggested that the IAA-JA crosstalks could account for the elicitation of the immune system by altering JA homeostasis (Nagpal et al. 2005; Gutierrez et al. 2012; Machado et al. 2016). Other plausible mechanisms of indole action have been speculated: the presence of a bacterial-like indole-binding protein in plants (Cofer et al. 2018) and the conversion of indole to an auxin-signalling inhibitor (Bailly et al. 2014). Given the central position of the indolic backbone in the biosynthesis of defence and hormonal metabolites, in addition to the growing interest in indole as an inter-organismal signal, one can expect further details on the detection of this common VOC in a near future.

Furthermore, a group of linear, unsaturated six-carbon molecules, commonly termed green-leaf volatiles (GLVs) as they are released from damaged leaves (the typical smell from fresh cut grass), have been long described to elicit plant responses (Scala et al. 2013; Ameye et al. 2018). The aldehydes (E)-2-hexenal and (Z)-3-hexenal, its reduced alcohol (Z)-3-hexen-1-ol and ester form (Z)-3-hexen-1-yl acetate interestingly originate, like JA, from the lipoxygenase pathway. In a similar manner, GLVs are also emitted from intact leaves under herbivory stress. Taken

together, these compounds can remotely trigger plant defence or prime the basal immune system. Despite their chemical proximity to JA, the mode of perception of GLVs appears distinct from JA signalling and is yet not resolved. Several reports described GLVs capable of depolarizing the plasma membrane, an event relayed by cytosolic calcium signalling (Asai et al. 2009; Zebelo et al. 2012) and putatively MAP kinase activation (Engelberth et al. 2013; Dombrowski and Martin 2018). On the other hand, a sum of work also described GLVs treatments to cause a rather unspecific change in the cellular redox balance (Davoine et al. 2006; Yamauchi et al. 2015; Scala et al. 2017). Until further evidence clarifies how GLVs do mediate their danger signal to plant recipients, it appears that a direct ligand–protein bond is not the favoured scenario.

As a consequence, the nebulous mode of action of GLVs, admittedly true airborne defence signals, opens a Pandora's box of troubles to pinpoint VOCs perception mechanisms. Deeper work employing a set of chemically diverse volatile cues will be required in order to direct the search for VOC perception into either countless one-to-one compound recognition systems, a family of proteins dedicated to distinguish a specific group of molecules, or unspecific chemical stress events generating a central response program. In any case, the possibility that composite volatile blends, especially emitted by microbes, elicit miscellaneous mechanisms remains.

Perhaps one of the most studied family of plant volatiles is the terpenes. Terpenes and terpenoids have been shown to be central to the attraction of pollinators (Knudsen et al. 1993; Dudareva and Pichersky 2000), to mediate indirect defence by recruiting beneficial organisms such as nematodes (Ali et al. 2011, 2012) and parasitoids (Vet and Dicke 1992; Hare 2011) or deter herbivore insects (Huang and Osbourn 2019). Several lines of evidence also suggest that terpenes elicit the innate immunity (Arimura et al. 2000, 2012; Godard et al. 2008; Cascone et al. 2015) and that the defensive message can be conveyed from plant to plant in analogy to GLVs (Kishimoto et al. 2005, 2006; Muroi et al. 2011; Riedlmeier et al. 2017). However, the dual biosynthetic origin of terpenes, deriving either from the chloroplastic methylerythritol phosphate/deoxyxylulose phosphate pathway or the cytosolic mevalonate pathway, in addition to the myriad of involved synthetic and modifying enzymes (terpene synthases and cytochromes P450) result in an outstanding chemical diversity (Degenhardt et al. 2009b; Boutanaev et al. 2015; Pichersky and Raguso 2018; Vattekkatte et al. 2018). With more than 40,000 known chemical structures to date, terpenes are often presented as the most diverse group of natural compounds on the planet. Although both terpenes biosynthetic pathways appear in some cases exclusive, terpenes are produced in virtually all organisms (Yamada et al. 2015b) and recently, they were proposed as universal communication cues (Schmidt et al. 2017). The plant responses triggered by terpene emissions encompass JA and SA signalling (Kishimoto et al. 2006; Riedlmeier et al. 2017) routes and lead to resistance against microbial pathogens. If it is established that terpenes induce transcriptional regulation, yet there is no available data on proteins mediating the general sensing of terpenes. The variety and net structural differences between these chemicals do not argue for a universal receptor type. Nonetheless, investigating how

plant mutant backgrounds disabled in JA or SA signalling respond to terpenes stimuli, as performed by Kishimoto and colleagues (Kishimoto et al. 2006) will certainly help in cornering essential components of the transduction machinery. An inspiring example of how terpenes might be sensed in the plant nucleus is described hereunder.

Finally, a range of volatile chemical perturbators might simply enter or hinder predetermined biosynthetic routes and ultimately imbalance their homeostatic status (Alméras et al. 2003; Dorokhov et al. 2012; Meldau et al. 2013; Aziz et al. 2016). Remarkable examples are the fine-tuning of the oxidative metabolism necessary to maintain optimal fitness, disturbed by isoprene (Loreto and Velikova 2001; Jardine et al. 2013), and the entry of sulphur-containing VOCs into sulphur metabolism (Meldau et al. 2013; Aziz et al. 2016). Whether such disequilibrium could ultimately weigh on the defence-to-growth ratio is not fully resolved, but these examples of rather non-specific elicitation of plant responses demonstrate how careful researchers have to be when envisaging proteins dedicated to volatilomes perception.

5.8 Achieving VOC Binding Protein Identification Is Within Reach

In this day and age, the development of high-throughput technologies to monitor plant reactions has tremendously eased the connection of diverse stimuli to protein networks. The appearance of microarray platforms (and by association Next Generation Sequencing methods), for example, has been instrumental in realizing the interdependence of discrete genetic modules overarching hormonal responses, defence layers or complex biosynthetic routes. The spatio-temporal dynamics of the plant transcriptome is extremely sensitive to even minor modifications of the plant environment, and this is not surprising that the sum of studies on VOCs reported thus far rather led to a confusing sum of plant transcriptional changes than to the identification of a common recognition process. Microbes emitting VOCs enhancing plant growth often elicit JA/ET-dependent innate immunity, as exemplified by the well-studied *Pseudomonas simiae* strain WCS417r (Ryu et al. 2003, 2004; Kwon et al. 2010; Zamioudis et al. 2013). Interestingly, the priming effect is of similar extent to the priming events triggered by direct contact with the microbe, as well as supported by ET/JA and auxin signalling pathways, eventually granting an enhanced resistance towards upcoming pathogens to the subjected plant (Verhagen et al. 2004; Stringlis et al. 2018). Follow-up work on the WSC417r–Arabidopsis interaction revealed that the R2R3-MYB-like transcription factor MYB72 was an early response gene integrating both volatile and non-volatile input into an ET-dependent transcriptional cascade. Further involvement of the iron homeostatic mechanism and the identification of a ISR-specific beta-glucosidase (BGLU42) resulted from in-depth analysis of the interaction, hence illustrating

the importance of genetically and biochemically decorticating the plant health-promoting properties of microbial emissions (Van der Ent et al. 2008; Zamioudis et al. 2014, 2015). Based on transcriptomic approaches and, by extension, on the analysis of plant mutants impaired in components or reporter constructs of the elusive VOCs-responsive pathways, a clearer picture of the molecular events downstream VOCs stimuli perception emerged. Despite this achievement, the means by which the plant recognizes WCS417r volatile blends remain unknown.

Whole genome association mapping is another powerful technology facilitated by the ever-lower costs of sequencing techniques. The association between observable, quantitative traits and single-nucleotide polymorphisms in a set of genetic variants allows for the robust identification of gene spaces explaining the trait variation. At least one genome-wide association study (GWAS) on Arabidopsis ecotype variants has been carried out to test for WCS417r plant growth-promotion (Wintermans et al. 2016). Although this work was not especially designed towards pinpointing VOC-related loci, the simple experimental setup used did not distinguish between volatile or non-volatile signals. This work failed at identifying genes linking the changes in root architecture triggered by the bacterial metabolites to the observed increase in shoot fresh weight; however, a restricted number of candidate loci involved in plant growth programs could be associated with this quantitative trait. Among the ten genes associated with growth enhancement and four genes with lateral root number, no obvious candidates for a receptor-like or defence-associated protein were identified. Still, the authors extended their analysis up to 10 kb around the associated polymorphisms, thus yielding a larger list of genes, including numerous uncharacterized ones, putatively involved in the VOC response. Even though the multitude of stimuli received by the plants in this particular study did not ease the description of loci categorically linked to VOC perception, future GWAS strategies focusing on a discrete number of volatile signals will be valuable in pinpointing genes contributing to the mechanism. The success of GWAS approaches certainly resides in the choice of the quantitative trait to monitor, and beyond measurements of plant architecture, one can easily imagine that more steady and precise outputs, such as ISR-dependent gene expression, metabolite profiling or pathogen resistance could represent ideal phenotyping tools to gain information on the functional recognition of volatile species.

Again, defining a ligand-to-protein methodology seems the most straightforward way to genetically isolate VOC-binding proteins. Although a variety of microbial compounds unequivocally causing quantifiable plant responses or leading to obvious phenotypes have been reported in the recent literature, there is to date no report of their utilization in classical genetics. For example, early-discovered molecules like 2,3-butanediol, acetoin (Ryu et al. 2003, 2004; Farag et al. 2006) or 1-octen-3-ol (Kishimoto et al. 2007) were used in a plethora of studies as prominent bacterial or fungal metabolites to dissect the plant response to volatile stimuli (Han et al. 2006; Cho et al. 2008; D'Alessandro et al. 2014; Kong et al. 2018). The vast majority of authors though investigated the effects of VOCs in plants carrying lesions in genes previously known to be central to defence or growth pathways, but hardly yielded novel mutations. The power of plant mutagenesis, and particularly in Arabidopsis, is

undebated and directly applicable to volatile treatments. The canonical experiment carried out by Bleecker et al. at the end of the 1980s identified the first ethylene receptor ETR1 from a mutagenized seed pool screened for insensitivity towards ET (Bleecker et al. 1988). It is therefore intriguing that more than 15 years after the first reports on 2,3-butanediol, no mutant was described as "butanediol-resistant". It is conceivable that the robustness of the plant growth responses towards VOCs may not grant sufficient confidence in selecting mutations in large screen populations as the increase in biomass might greatly vary between individuals and might depend on the mode of volatile delivery in simple Petri dish setups. However, without considering the existence of receptors binding volatile species, if the genetic basis of the plant response to these chemicals is genuine, it can be uncovered by a combination of careful experimental designs and well-established protocols.

Future efforts in identifying proteins partaking into the VOC signal transduction should therefore follow a complete set of top-down approaches. First, it is essential to predefine the monitored outputs that lead to the response of interest. Gene activity, reporter fusions or growth parameters known to be influenced by volatile exposure have been extensively described and can readily serve in the measurements of the plant response. Second, describing a single or a subset of chemical species will indisputably avoid masking effects generated by the multifarious nature of biogenic volatile blends. Although it is generally admitted that VOC mixtures are more effective than single compounds (Ueda et al. 2012) in eliciting plant responses, breaking down the original group of compounds to a meaningful set of active ingredients will ease downstream processes. Third, in order to confirm or infirm the potency and/or specificity of the compounds of interest, structure–activity relationship studies are most informative. Defining a pharmacophore is key in associating the compound of interest to families of known active substances or recognizing putative binding pockets in proteins. Once these experimental parameters are set, it is up to the experimenter to choose the appropriate technology to corner candidate genes or proteins.

This classical workflow has recently proven extremely successful. In a screen for terpenes and GLVs that trigger stress-responsive gene expression using Nicotiana plants and cell culture lines, Nagashima and colleagues identified sesquiterpenes as *bona fide* inducers (Nagashima et al. 2019). Based on the quantitative PCR results of three defence-related reporter genes and appropriate MeJA and SA control treatments, the authors showed that caryophyllenes steadily activate the expression of the pathogenesis-related protein gene Osmotin in BY-2 cells. Following time- and dose-dependent Osmotin induction by (E)-β-caryophyllene, α-caryophyllene and caryophyllene oxide, further validated by promoter-fusion construct assays, Nagashima *et al.* showed that the *in planta* response to caryophyllenes was essentially occurring in leaves, in line with previously published work (Rasmann et al. 2005; Degenhardt et al. 2009a). They next opted for biotinylation of the caryophyllene to achieve streptavidin-assisted pull-down assays and recovered a 120 kDa TOPLESS-like (TPL) transcriptional corepressor from tobacco leaf extracts. Subsequent cloning and overexpression of Nicotiana TPLs and TPL-GFP fusions in tobacco plants and cell cultures demonstrated that the transcription

regulators reside into nuclei, respond to caryophyllenes and ultimately regulate Osmotin expression. Therefore, this work can be considered the first and most consistent genetic and biochemical demonstration of the long-sought VOC perception outside of hormonal regulators. Interestingly, TPL proteins have been characterized as components of the JA- and IAA-mediated signalling pathways recruiting JAZ proteins (Szemenyei et al. 2008; Pauwels et al. 2010; Pérez and Goossens 2013). The mechanics by which this particular terpene seems to be recognized and transduced hence parallel the mode of perception of volatile phytohormones. A concept in which a context-specific transcriptional repression could be achieved by the recruitment or release of transcription factors by TPL proteins upon VOC binding is seducing, especially owing to the broad interactome of such proteins (Causier et al. 2012), and might prove valid for more volatile cues to be discovered.

5.9 Concluding Remarks

Plants are continuously exposed to numerous stresses ranging from injury and environmental factors to disease and dehydration. In order to survive to the next generation, they must amend their physiology in response to such threats. Whether plants are able to proactive decision-making is currently under debate; however, their ability to sense subtle alterations in their environment and mount appropriate responses is key to their evolutionary success. There is little doubt that organic volatile cues represent a major input into the cellular programs dedicated to react to these changes for they are indicative of active living organisms susceptible to interact with the plant body. Nevertheless, plants are, *in natura*, surrounded by a multitude of VOCs and it remains very challenging to identify meaningful molecules that could serve as *bona fide* semiochemicals in ecological relationships. Thus far, the large majority of reported experimentations were rather designed to uncover if plants perceive such signals. After more than a decade and a growing number of independent laboratories reporting converging results, it seems established that the entire plant kingdom possess this faculty. Moreover, the emission and reception of VOC-based information appears more and more as an ancient, inherited function spread throughout the whole tree of life. Volatile interactions can easily be appreciated as a core module of the ecological webs governing the behaviours of species and individuals in a competitive environment. Therefore, as plant bodies are essentially decentralized, in contrast to animal sensory organs and decision centres, the phyllosphere and rhizosphere of plants are no less than a prodigious interface to monitor and integrate signals from the outside. The physico-chemical properties of volatile organic compounds have been exploited by plants to support essential communication functions that coordinate development, growth and defence. It is hence reasonable to envisage that, if VOC recognition granted a fitness benefit to plants through evolution, the molecular means to achieve such recognition lie into the recruitment of ancestral gene sets, the modulation of central enzymatic pathways and/or innovation from existing perception systems. In order to increase our

understanding of volatile-mediated interactions in plants, further efforts should then be made in three directions. First, the definition of reliable VOC-induced traits that will serve as the basis for genetic approaches. Second, the classification of individual or families of VOCs into bioactive groups leading to common plant responses. Third, the establishment of systematic protocols to monitor large-scale plant transcriptome and proteome changes upon VOC stimulation. By standardizing experimental procedures and pooling their output into an exploitable platform, the scientific community interested in deciphering plant volatile interactions could effortlessly exploit the mass of Arabidopsis-centred tools that have been developed in the past two decades to pinpoint candidate genes and, perhaps, trigger the rise of volatilomics.

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Chapter 6
Contribution of Bacterial Volatiles to Chemical Ecology

Rouhallah Sharifi and Choong-Min Ryu

Abstract Bacterial volatile organic compounds (VOCs) mediate biological interactions. Microbes acquire information from their niche and affect the physiology and behaviour of their competitors using the highly diffusible VOCs. VOCs act in both positive and negative interactions. Microbes and eukaryotic host have evolved specific sensing mechanisms to perceive and respond to VOCs from their symbiotic partner. Some microbes have also acquired volatile biosynthesis genes from their symbiotic partner via horizontal gene transfer during their co-evolution. VOCs reduce the cost of antagonist interactions, as they prevent close conflicts by repelling competitors or killing them over a long distance. VOCs also play an important role in predator–prey interactions. Altogether, VOCs are a powerful tool for improving the competence of the emitting bacteria in their ecological niche. Airborne communication determines the complex relations within an ecosystem in a dynamic and sophisticated manner. VOCs are multifaceted compounds and a promising source of eco-friendly agrochemicals. Further research is needed to investigate and exploit VOCs in natural ecosystems.

Keywords Agrochemical · Ecosystem · Microbial robustness · Volatile organic compounds

6.1 Introduction

Microbial populations comprise individuals of same species. In nature, they live with other populations forming complex biological communities. Coexistence could be advantageous or detrimental for microbes; for example, microbes release enzymes,

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chelates and other public good compounds, which benefit both the producer and the recipient (Niehaus et al. 2019). Additionally, microbes degrade recalcitrant compounds, which act as substrates for other species. However, microbes need to compete with each other for nutrients in a source-limiting environment. Theoretically, in a nutrient-rich environment without any space limitation, microbes grow at an exponential rate. However, such ideal conditions are not common in nature. In complex ecological niches such as rhizosphere and mammals gut, microbial population and their demand is high, whereas resource availability is mostly limited and often heterogeneously distributed (Hill and Jones 2019; Jones et al. 2013). Even in rich media, microbes may suicide or have negative effect on each other (Coyte et al. 2015; Ratzke et al. 2018). Host organism also extrinsically regulated microbial populations. Overall, this negative or competitive effect supports stability of microbial communities (Coyte et al. 2015). Therefore, only a given number of microbes can colonise a shared ecological niche, which is referred to as the carrying capacity of that niche (Roller and Schmidt 2015). Carrying capacity indicates the maximum number of organisms that a niche can sustain. Some nutrient-rich zones have higher carrying capacity than others; for example, the rhizosphere, especially hotspots in the rhizosphere such as the root elongation zone, has a high carrying capacity. Because of the above-mentioned limitations, microbial populations follow logistic growth under natural conditions. Parameters of logistic growth depend on the carrying capacity of the niche and growth strategy of microbes [zymogenous (r-strategy) or autochthonous (k-strategy)] (Langer et al. 2004; Roller and Schmidt 2015). In a natural environment, microbial populations not only adjust their own growth using quorum-dependent strategies, but also modulate the growth of other microbial species via other strategies. Competition is considered as the main ecological relationship in the microbial world (Li et al. 2019). Microbes develop two groups of competitive strategies, explorative competition and interference competition (Ghoul and Mitri 2016), also known as competitive exclusion and antagonistic exclusion, respectively (Ghoul and Mitri 2016). In the explorative competition strategy, microbes acquire the ability to quickly and effectively colonise ecological hotspots, consume diverse nutrient sources, uptake source-limiting nutrients such as iron and phosphate, consume the by-products of other microbes and move to new nutrient-rich environments. In the interference competition strategy, microbes synthesise and secrete metabolites such as antibiotics, bacteriocins, quorum quenchers, growth inhibitors and reactive oxygen species to reduce or suppress the growth of other organisms. Thus, genes related to competition mechanisms are highly diverse, and constitutive expression of these genes would challenge microbial growth (Dragset et al. 2019; Lambert and Kussell 2014; Stubbendieck and Straight 2016). Additionally, microbes have developed strategies to sense competition and the type of competitors. Some microbes can sense resource limitations, interfering metabolites released by other microbes and damage in related species (Abrudan et al. 2015; Jones et al. 2017; Leinweber et al. 2018). This phenomenon is similar to the perception of microbe-associated molecular patterns (MAMPs) and

damage-associated molecular patterns (DAMPs) in plants and animals (Heil and Land 2014).

Microbes can make fast and accurate decisions with the lowest resource investment, given the availability of comprehensive information from their niche. Sources of information can be divided into three categories. Concentration gradient of any compound can be a source of information, which can help determine its origin. Cues are compounds inadvertently secreted or emitted by an organism that serve as a source of information for the receiving organisms. Signals deliberately produced by an organism to change the behaviour of related or unrelated species are also a source of information. Some microbes also eavesdrop on the signals of other microorganisms to monitor their status.

Microbes exploit volatile and non-volatile compounds as sources of information in their ecological interactions. However, volatile compounds have an important advantage over non-volatile compounds, as these can diffuse through water, air and soil over long distances and expand the span of available information (Sharifi and Ryu 2018b; Simpraga et al. 2016). Microbes may prevent close conflict by repelling competitors via the release of long-distance moving infochemicals. Moreover, infochemicals prime competition-related genes in receiver microbes to induce faster and stronger response in upcoming conflicts. Bacterial volatile organic compounds (VOCs) may act as cues or signals, based on their nature and the type of interaction. Microbes including bacteria release a blend of volatile compounds belonging to different classes of chemicals at different concentrations. Several reports focus on VOCs as signature compounds for the fast identification of bacterial species (Choudoir et al. 2019; Lewis et al. 2017; Rees et al. 2018). However, the profile of VOCs varies with the age of bacteria, composition of nutrient media and type of ecological interaction (Asensio et al. 2007; Choudoir et al. 2019; Insam and Seewald 2010; Schmidt et al. 2016). We emphasise that variation in the VOC profile of a bacterium strain is informative per se. Some volatiles are shared by higher taxa. For example, 1-octone-3-ol, which has a mushroom-like odour, is common among some fungal phyla, whereas geosmin, which has an earthy flavour, is common in Streptomyces. These volatiles inform the receiver organism about its neighbours. Release of some volatiles is affected by environmental and nutritional conditions, which is informative to the microbiome of a specific niche (Asari et al. 2016; Blom et al. 2011). Some VOCs are released specifically as a consequence of ecological interactions. The production of sodorifen in *Serratia plymuthica* increases after exposure to VOCs from *Fusarium culmorum* (Schmidt et al. 2017). Rybakova et al. (2017) showed that the production of two volatiles, 2-methyl-1-butanol and isoamyl alcohol, increased in *Paenibacillus polymyxa*, whereas that of isoamyl acetate and durenol decreased noticeably upon exposure to *Verticillium longisporum*. In another study, the emission of dimethyl-hexa-decylamine (DMHDA) from *Arthrobacter agilis* increased 12-fold after interaction with *Medicago sativa* (del Carmen Orozco-Mosqueda et al. 2013). Moreover, discrete VOCs can react in headspace of separate microbial colonies grown in same place to form de novo volatiles. For example, schleiferon A is produced after a non-enzymatic reaction between acetoin and 2-phenylethylamine in the headspace of *S. plymuthica* and *Staphylococcus*

delphini co-culture (Kai et al. 2018). Therefore, VOCs are dynamic compounds that represent the taxonomy, habitat, nutritional status and biological interactions of microbes.

In this chapter, we explain the role of VOCs in different types of biological interactions such as mutualism, protocooperation, commensalism, amensalism, antagonism, competition and predation. We also discuss the role of VOCs in the survival of microbes in biological interaction networks.

6.2 Role of VOCs in Ecological Interactions

6.2.1 Mutualism

Symbionts live together and coordinate their functions as a holobiont during long periods of co-evolution. Consequently, symbionts learn to sense and respond to cues and signals from their partner. Bark beetle (*Ips typographus*) developed specific olfactory sensing neurons in its antennae to sense volatiles released by its ophiostomatoid fungal symbiont (Kandasamy et al. 2019). Beetles are attracted by the food recourse colonised by their symbiont but not by saprophytic organisms (Fig. 6.1). Symbionts acquired the de novo feature of chemically communicating with their partner. In some cases, microbial symbionts emit volatiles that act as pheromones for their animal partner. Symbiotic fungi emit (−)-verbenone and 2-methyl-3-buten-2-ol as bark beetle anti-aggregation and aggregation pheromones, respectively, to facilitate their dispersal (Kandasamy et al. 2016, 2019; Zhao et al. 2015). Bacterial symbionts of ant (*Atta sexdens rubropilosa*) emit ant trail and alarm hormones belonging to the pyrazine group to attract ants and initiate a symbiotic relationship (Silva-Junior et al. 2018). Pyrazine compounds are also released by *Pseudomonas* spp., which act as sex pheromones for the tree frog symbiont, *Boana prasina* (Brunetti et al. 2019).

Symbionts develop specific features to communicate with their partner using chemical signals via mechanisms such as adaptation, natural selection and horizontal gene transfer. The genome of the entomopathogenic fungus *Metarhizium* spp. harbours terpene synthase genes, which are most likely acquired from their bacterial symbionts. The expression of these genes, known as bacterial TPS-like genes (BTPSL), leads to the production of sesquiterpenes such as corvol ether (Jia et al. 2019).

6.2.2 Protocooperation

Protocooperation occurs between two organisms that can live separately but benefit from each other in cooperation. Probiotic–host interaction is a type of protocooperation, and plant growth-promoting rhizobacteria (PGPR) are considered

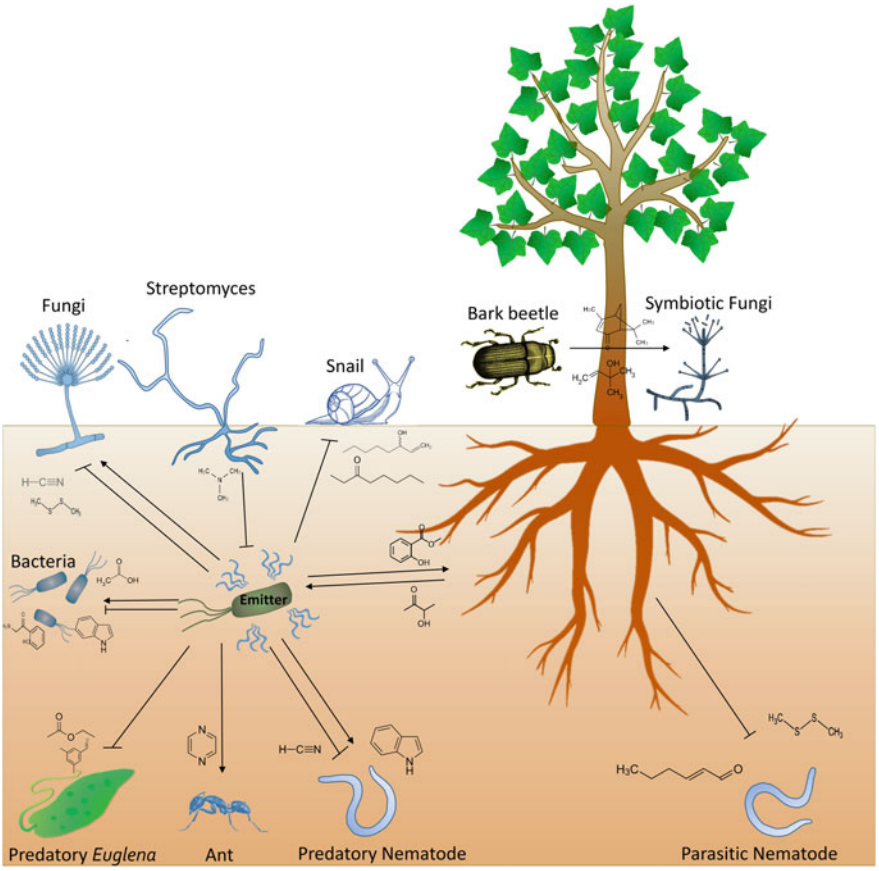

Fig. 6.1 Bacterial volatile compounds play important roles in biological interactions. They may be taken up as food source, regulate growth or change behaviour of neighbour organisms. This figure depicted some of these interactions between organisms in different taxonomic groups. Details can be found in main text

as the main plant probiotics (Menendez and Garcia-Fraile 2017; Woo and Pepe 2018). PGPR support plant health and growth, and plant build a well-developed and nutrient-rich habitat for them. Several comprehensive reviews have been published on BVC-mediated plant–PGPR protocooperation (Chung et al. 2016; Piechulla et al. 2017; Sharifi and Ryu 2018a, b; van Dam et al. 2016). Another example of protocooperation is the yeast–fruit fly interaction. Fruit flies feed on yeast but also help in yeast dispersal and outbreeding. VOCs such as acetic acid, acetoin and 3-methyl-1-butanol released from yeast-colonised fruits attract fruit flies. Yeast volatiles improve fruit fly attraction, larval development and oviposition (Becher et al. 2012).

6.2.3 Commensalism

Commensalism is an ecological interaction between two organisms, where one organism benefits the other, while the other organism neither benefits nor harms its partner. VOCs such as terpenes, dimethyl sulphide (DMDS) and hydrogen cyanide (HCN) released by microbes are consumed as sources of carbon, nitrogen and sulphur by other microbes (Chinchilla et al. 2019; Hammerbacher et al. 2019; Scott et al. 2019; Wang et al. 2014). Some of the above-mentioned compounds are toxic at high concentrations. However, toxic compounds may act as growth stimulators at low concentrations, a phenomenon known as hormesis (Calabrese and Baldwin 2003). Furthermore, microbes can convert toxic volatiles to nutrient sources or remove them from the cell. *Fusarium solani* biodegrades HCN to form formamide, formate and ammonia are then consumed as a carbon or nitrogen source (Dumestre et al. 1997). *Grosmannia clavigera* consumes monoterpenes as a carbon source at a low concentration but effluxes excess amount via an ABC transporter (Wang et al. 2013). Turning threats into opportunities is a characteristic feature of ecological competence among microbes, which improves their ability to increase their population in a competitive niche.

6.2.4 Amensalism and Antagonism

In antagonism, one organism produces toxic compounds to exclude competitors. In amensalism, toxic compounds, considered as by-products of microbial metabolism and released into the environment, incidentally inhibit the growth of other organisms. Thus, there is no striking difference between antagonism and amensalism in microbial ecology. Antagonists release antimicrobial compounds such as antibiotics, bacteriocins and toxins to kill competitors and expand their area. A well-known example of amensalism is algal bloom, which can lead to eutrophication and oxygen (O_2) depletion, thus harming and killing several marine species (Riebesell et al. 2018). Post-harvest biocontrol yeasts also produce a large amount of carbon dioxide (CO_2) and deplete O_2 in a modified atmosphere and suppress the growth of other microbes (Contarino et al. 2019). Streptomyces release volatiles such as trimethylamine (TMA) and ammonia, which decrease the growth of neighbouring microbes (Fig. 6.1). These compounds increase the environmental pH to 9.5, which not only directly affects microbial growth but also reduces the solubility of metal ions such as Fe^{3+}. However, Streptomyces secrete siderophore desferrioxamine, which has a high affinity for iron, and therefore not only obviates iron starvation but also decreases the iron content of media. Addition of iron to the growth medium prevents the inhibition of growth by TMA and ammonia (Jones et al. 2017, 2019). *Salmonella typhimurium* releases hydrogen sulphide (H_2S), which makes the inherently cephalosporin-resistant enterococci completely susceptible to cephalosporin

and supports *S. typhimurium* colonisation in the intestine by removing competitor enterococci (Thomas-Lopez et al. 2017).

Several studies have reported the antagonistic effect of VOCs on the growth of fungi (Table 6.1) (Piechulla et al. 2017; Rojas-Solís et al. 2018; Wan et al. 2008) and bacteria (Agisha et al. 2019; Ajilogba and Babalola 2019; Kim et al. 2013; Rajer et al. 2017; Raza et al. 2016); however, this effect is often dose-dependent. VOCs are also reported to have a positive effect on microbial growth (Chen et al. 2015; Sharifi et al. 2013). Therefore, the aim should be to check the antagonistic effect of VOCs at their natural concentration in ecological studies. Some VOCs are toxic to other organisms at their natural concentration. VOCs released by *Bacillus amyloliquefaciens* NJN-6 inhibit the growth of several fungi but increase the abundance of certain bacteria such as *Proteobacteria*, *Firmicutes* and *Bacteroidetes* (Yuan et al. 2017). Profiling volatiles and the taxonomic position of fungi indicates that the response to bacterial volatile compounds varies among fungi. Several reports show that *Fusarium oxysporum* and *F. solani* are highly resistant to bacterial volatiles, whereas oomycetes are highly sensitive (Che et al. 2017; Hunziker et al. 2015; Van Agtmaal et al. 2018). Table 6.1 lists several antifungal VOCs and their effective concentrations. Furthermore, VOCs also negatively impact soil macrofauna. For example, DMDS, which has been patented as a new nematicide (de Boer et al. 2019), and 3-pentanol exhibit nematicidal activity against *Meloidogyne incognita* at concentrations of 176 and 918 μg/mL, respectively (Silva et al. 2018). Similarly, VOCs (*E*,*E*)-2,4-decadienal and (*E*)-2-decenal show negative effects on *Meloidogyne javanica* at concentrations of 11.7 and 20.4 mg/L, respectively (Caboni et al. 2012). The volatile compound (*E*)-2 hexenal also has a nematicidal effect in the field at a concentration of 500 L/ha (Lu et al. 2017). Volatiles 1-octene, 3-octanone and 1-octen-3-ol from *Metarhizium brunneum* exhibit negative effects on the snail species *Cornu aspersum* at low concentrations (Khoja et al. 2019). Additionally, volatiles from *Metarhizium* repel snails at a concentration of 1–5 μL per 9 cm Petri dish and show molluscicide activity at 10 μL per Petri dish. Moreover, a 20% solution of 3-octanone and 1-octen-3-ol could be sprayed on plants as a molluscicide without any phytotoxic effects (Fig. 6.1). These reports indicate that volatile compounds could be considered as a new source of pesticides in agriculture. However, VOCs are sensitive to evaporation and oxidation; therefore, the development of adequate technology is needed for the application of VOCs (Sharifi and Ryu 2018c). Micro- and nano-formulations are promising technologies to transfer these compounds effectively not only to the aerial plant parts but also to the root zone (Chariou et al. 2019; Sharifi and Ryu 2018c; Wang et al. 2019).

VOCs disperse over a wider area. Therefore, they may reduce the risk of close conflict, which needs development of contact-dependent inhibition strategies such as type 6 secretion system and other toxin delivery membrane proteins (Stubbendieck and Straight 2016). The synthesis and release of VOCs as growth inhibitors are also more cost-effective than the production of antibiotics (Avalos et al. 2019).

Table 6.1 List of bacterial volatile compounds and their concentrations effective against fungi

Volatile compounds	Emitters	Target organisms	Effective concentration	References
3-Methyl-1-butanol Phenylethyl alcohol 2-Methyl-1-butanol	*Pseudomonas chlororaphis*	*Ceratocystis fimbriata*	143–1143 μL/L	Zhang et al. (2019b)
1-(2-Aminophenyl) ethanone (EA) Benzothiazole	*Bacillus* spp.	*Peronophythora litchii*	146–170 mg/L	Zheng et al. (2019)
Dimethyl disulphide Methyl isovalerate	*Alcaligenes faecalis* N1-4	*Aspergillus flavus*	50–100 μL/L 200 μL/L	Gong et al. (2019)
2-Nonanone and 2-heptanone	*Bacillus amyloliquefaciens* L3	*F. oxysporum* f. sp. *niveum*	50 μL per I-plate	Wu et al. (2019)
2-Undecanone	*Bacillus* spp.	*Sclerotinia sclerotiorum*	1.8 μg/mL	Massawe et al. (2018)
2,4-Di-*tert*-butylphenol	*B. subtilis* CF-3	*Colletotrichum gloeosporioides*	200 μL of 1 M/L	Zhao et al. (2019)
2-Ethyl-1-hexanol	*Lysinibacillus* spp.	*Colletotrichum acutatum*	10 μL per plate	Che et al. (2017)
Dimethylhexadecylamine	*Arthrobacter agilis* UMCV2	*Botrytis cinerea* *Phytophthora cinnamomi*	100 μM	Velázquez-Becerra et al. (2013)
Benzothiazole Phenol, 4-chloro-3-methyl	*Bacillus velezensis* ZSY-1	*B. cinerea* *Alternaria solani*	80 μL	Gao et al. (2017)
Dimethyl trisulphide	*Pseudomonas putida* BP25	*Phytophthora capsici* *Rhizoctonia solani*	6.25 μg/cm^3	Agisha et al. (2019)
S-methyl methanethiosulphonate	*Pseudomonas* spp.	*Phytophthora infestans*	1 mg per 80 mL petri dish	Chinchilla et al. (2019)

6.2.5 *Predation*

Bacteria serve as prey for amoeba, nematodes, mites and other small predators. Predators exploit bacteria volatiles and their concentration gradient to identify prey-rich locations or to determine their favourite prey. Bacteria terpenoid volatiles play an important role in attracting their protist predators (Schulz-Bohm et al. 2017). The protozoan predator *Euglena* tracks the location of its prey, *Listeria*, via VOCs such as decanal, 3,5-dimethylbenzaldehyde and ethyl acetate (Fig. 6.1) (Gaines et al. 2019). However, VOCs may have negative effects on predators by killing or repelling them. The bacteriovorus nematode, *Caenorhabditis elegans*, senses a low concentration of indole and moves towards the indole-producing bacteria. However, indole concentration greater than 0.5 mM has a negative effect on oviposition and predatory performance of the nematode (Lee et al. 2017). A slightly higher indole concentration (1–2 mM) also has a negative effect on the predatory performance of *Bdellovibrio bacteriovorus* (Dwidar et al. 2015). This concentration is similar to that in the mammalian gut (Mun et al. 2017). HCN, which inhibits aerobic respiration, plays an important role in predator–prey interactions. The bacterium *Chromobacterium piscinae* releases 100–200 μM HCN, which decreases *B. bacteriovorus* motility and bdelloplast development (Mun et al. 2017). HCN-producing bacteria such as *Pseudomonas aeruginosa* and *C. piscinae* possess *cioAB* genes or their homologues, which encode cyanide-insensitive oxidases that confer immunity against high concentrations of HCN (Frangipani et al. 2014; Mun et al. 2017). *P. aeruginosa* and pure HCN at levels similar to that released by the emitter bacterium kill the predatory nematode *C. elegans* (Gallagher and Manoil 2001).

6.3 Role of VOCs in Microbial Robustness

Microbes support their survival and maintain their population in a dynamic ecosystem by quickly adapting to changes, moving towards rich zones and developing tolerance to biotic and abiotic stresses. Infochemicals such as VOCs play important roles in facilitating the above-mentioned phenomena. VOCs can also change several ecologically important phenotypes and behaviours of neighbouring microbes such as motility, root colonisation, biofilm formation, sporulation and virulence (Table 6.2). Transcriptome and proteome analyses demonstrate global changes in the cellular response of microbes to VOCs (Chinchilla et al. 2019; Garbeva et al. 2014; Kim et al. 2013; Raza et al. 2016; Tahir et al. 2017). It is reasonable to consider a master regulatory system such as GacS/GacA and quorum sensing (QS) system in the event of global changes in gene expression or the phenotype of an organism. These two-component sensor regulator/response activator systems regulate several intracellular and cell-to-cell mechanisms at the transcriptional and post-transcriptional levels. Several reports show that VOCs affect these global regulatory systems and

Table 6.2 Effects of VOCs and fungal volatile compounds on different microbial features

Microbial feature	BVC	Emitter	Receiver	Mode of action	References
Motility	Dimethylhexadecylamine	*Arthrobacter agilis* UMCV2	Bacillus spp. *Pseudomonas fluorescens*	Swarming motility genes	Martínez-Cámara et al. (2019)
	Benzaldehyde 1,2-Benzisothiazol −3 (2H)-one 1,3-Butadiene	*Bacillus* spp.	*Ralstonia solanacearum*	Chemotaxis, swarming, swimming and twitching motility	Tahir et al. (2017)
	Decyl alcohol 3,5,5-Trimethylhexanol	*Bacillus* spp. 15 D13	*Xanthomonas oryzae* pv. *oryzae*	Swarming swimming	Xie et al. (2018)
	2,3-Butanedione	*Bacillus subtilis*	*Escherichia coli*	Swimming motility genes	Kim et al. (2013)
Biofilm	Acetic acid	*B. subtilis*	*B. subtilis*	ywbHG gene in biofilm formation	Chen et al. (2015)
	2-Aminoacetophenone	*Pseudomonas aeruginosa*	*Vibrio fischeri*	LuxR response regulator	Kviatkovski et al. (2015)
	2-Nonanone, 2-heptanone, 2-undecanone, DMDS	*Pseudomonas* spp. *Serratia* spp.	*Agrobacterium tumefaciens*	Biofilm formation and cell survival in mature biofilms	Plyuta et al. (2016)
Virulence	Volatile blend	*Bacillus* spp.	*Sclerotinia sclerotiorum*	Oxalic acid accumulation	Massawe et al. (2018)
	2,4-Di-*tert*-butylphenol	*B. subtilis* CF-3	*Colletotrichum gloeosporioides*	Ergosterol content	Zhao et al. (2019)
	Benzothiazole	*B. subtilis* CF-3	*Monilinia fructicola*	Ergosterol content	Zhou et al. (2019)
Enzymes	2,3-Butanediol Acetoin	*Pectobacterium carotovorum* subsp. *carotovorum*	*P. carotovorum* subsp. *carotovorum*	Pectate lyases expression and activity	del Pilar Marquez-Villavicencio et al. (2011)
	Benzothiazole	*B. subtilis* CF-3	*M. fructicola*	Pectinase and cellulase activity	Zhou et al. (2019)

Toxin	Volatile blend	*Alcaligenes faecalis* N1-4	*Aspergillus flavus*	Aflatoxin biosynthesis	Gong et al. (2019)
	Volatile blend	Yeast species	*Aspergillus ochraceus Aspergillus carbonarius*	Ochratoxin A	Farbo et al. (2018)
Antibiotic resistance	Volatile blend	*B. subtilis*	*E. coli*	AmpicillinTetracycline	Kim et al. (2013)
	Volatile blend	*Bacillus ambifaria*	*E. coli*	GentamicinKanamycin	Groenhagen et al. (2013)

vice versa, i.e., the biosynthesis of VOCs can also be affected by these systems (Chen et al. 2015; Cheng et al. 2016; Ossowicki et al. 2017; Shimada et al. 2013). Some VOCs such as indole, 2-aminoacetophenone, acetic acid, TMA and pyrazines act as cell–cell communication signals to modify microbial behaviour (Chen et al. 2015; Jones et al. 2017; Kviatkovski et al. 2015; Lee et al. 2015; Shimada et al. 2013). QS systems regulate several genes responsible for important phenotypes such as biofilm formation, virulence, secretion and antibiotic resistance. Therefore, some phenotypes altered by the BVC treatment may represent the effect of VOCs on the QS system. For example, bacteria can share antibiotic resistance genes rapidly in the biofilm structure, and volatile QS inhibitors can alter antibiotic resistance in bacteria. QS inhibitors are considered as a new source of antimicrobial drugs (Chernin et al. 2011; Paul et al. 2018). DMDS inhibits QS by suppressing the production of *N*-acyl homoserine lactones (AHL) in some *Pseudomonas* strains (Chernin et al. 2011). VOCs mediate the inhibition of the QS system in competing neighbours, which seems to be a widespread phenomenon in bacteria ecology (Chernin et al. 2011; Plyuta et al. 2016). Thus, volatile cell–cell communication signals have an important effect on bacterial biology and robustness.

TMA can be considered as a cell–cell communication signal, as it induces explorative competition in Streptomyces by reaching new nutrient-rich environment. In this phase, aerial mycelium does not produce spores but spreads rapidly over the medium to access the nutrient-rich environment. Explorative competition is initiated by nutrient starvation in the media, and TMA acts as a signal to change the growth phase in original and neighbouring colonies (Jones et al. 2017, 2019). Several bacterial species use the flagellum to initiate the explorative phase and move towards nutrient-rich environment or to repel dangers. VOCs induce or interfere with the flagellum-dependent and independent movement such as swimming, swarming and twitching motility in bacteria (Kim et al. 2013; Martínez-Cámara et al. 2019; Tahir et al. 2017; Xie et al. 2018). Alteration of chemotaxis and motility has a great impact on microbial robustness by limiting their access to nutrient-rich sources and ecological hotspots. Thus, some bacteria may exploit VOCs to suppress the motility and explorative ability of competitor microbes.

VOCs released by rhizobacteria improve root proliferation and change root secretion (Bailly et al. 2014; Yi et al. 2016). Rhizobacteria inhabit the rhizosphere, and shaping the rhizosphere implies shaping the habitat of rhizobacteria and increasing their robustness. *B. subtilis* emits 2,3-butanediol, which increases its robustness in the rhizosphere. *B. subtilis* strain overexpressing 2,3-butanediol not only survives for a longer duration in the rhizosphere of pepper plants but also reduces the colonisation of pepper rhizosphere by *Trichoderma* spp. Exudates from plants treated with 2,3-butanediol or the overexpressor strain inhibit the growth of several organisms (Yi et al. 2016). This shows that VOCs can alter plant physiology and root exudates in favour of their emitter organisms (Peng et al. 2019). Bacteria produce 2,3-butanediol and its precursor acetoin to regulate the intracellular pH of bacteria under acidic or hypoxic condition (Vivijs et al. 2014). Bacteria shift to mixed acid fermentation in hypoxic or anaerobic condition and synthesise ATP, but decrease cellular pH by producing acetic, lactic, succinic and formic acids. However,

pH-stimulated enzymes activate the acetoin/butanediol fermentation pathway, increasing the cellular pH and improving bacterial survival under the acidic condition. This phenomenon supports bacterial survival in the ecosystem with high variation in pH and O_2 supply (Vivijs et al. 2014, 2015). However, the acetoin/2,3-butanediol pathway is not an energy producing pathway. Instead, overexpression of this pathway eliminates pyruvate, two molecules of which are needed to produce one molecule of acetoin, from energy production under anaerobic conditions. Thus, overexpression of butanediol may increase the lifespan of bacteria but decrease their health span. Health span is defined as the time period that an organism is alive, healthy and free of frailties (Sonowal et al. 2017). Health span in bacteria could probably be measured by factors such as ATP level, qCO_2, motility and nutrient uptake rate. Xiao et al. (2010) reported that carbon sources that increase acetoin production do not support the growth of *Bacillus pumilus* ATCC 14884 and vice versa. Further investigation is needed to understand the effects of VOCs such as 2,3-butanediol on the health span of bacteria. Some bacterial cells, known as persister cells, reduce their metabolism in stress condition to increase their lifespan (Zhang et al. 2019a). Bacteria may exploit VOCs to modulate resuscitation of their competitors. *Escherichia coli* uses indole to inhibit *P. aeruginosa* persister cell resuscitation (Zhang et al. 2019a). In contrast, indole from commensal bacteria improves health span factors such as locomotion and pharyngeal pumping in *C. elegans* (Sonowal et al. 2017). Indole is a common volatile in rhizobacteria and plants, which play important role in intra- and inter-kingdom relationship in the phytosphere (Bailly et al. 2014; Bhattacharyya et al. 2015; Erb et al. 2015). Further research is needed to characterise the roles of indole in lifespan and health span of phytosphere organisms.

6.4 Conclusion

There are two opinions on synthesis and function of VOCs; some suppose that VOCs are by-products of the primary metabolism of microorganisms. However, the others argue against this claim because VOCs play a major role in ecological interactions and organism survival. In our opinion, both arguments are true. Most of the VOCs are derived from primary metabolism and released into the environment for several reasons including metabolic waste. VOCs, including those discarded as a waste, can act as nutrient sources and cues. More importantly, VOCs provide a real-time overview of the emitter physiology. Neighbouring microbes acquired ability to perceive the meaning of informative VOCs or consume them as a nutrient source during coexistence or co-evolution by the force of natural selection.

Each microbial strain releases a highly variable blend of VOCs, depending on the spatiotemporal ecological conditions. Thus, this variation is not random but originates from the ecosystem dynamics and could be meaningful for neighbouring microbes. Informative VOCs are a double-edged sword for both the emitting and receiving organisms. Emitter organisms can attract or repel their predators, based on

the presence or absence and concentration of specific volatiles. VOCs also notify competitors to prepare for a strong opposition. VOCs can interfere or directly disrupt the physiology and survival of competitors. Thus, VOCs act as an information source, attractant, repellent and nutrient source for the receiver organism.

Although volatile blends of different microbial species show more or less similar effects on target organisms under laboratory conditions, each volatile compound has a relatively specific target. Some VOCs directly inhibit pathogenic fungi (Chinchilla et al. 2019), and some induce plant defence against them (Sharifi and Ryu 2016). VOCs have multisite targets (Chinchilla et al. 2019), which reduce the probability of arising resistant races of target organisms by modifying target molecules. Resistant strains efflux toxic VOCs or transform them into safe compounds via degradation (Dumestre et al. 1997; Wang et al. 2013). Altogether, VOCs are promising candidates for new pesticides. However, their effects need to be evaluated on non-target organisms (Sharifi and Ryu 2018a). Although efforts are currently underway to formulate VOCs and apply them in the field as agrochemicals, successful development of these agrochemicals requires a comprehensive scientific background based on ecological data.

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Chapter 7
Bacterial Volatile-Mediated Plant Abiotic Stress Tolerance

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Abstract Plant growth-promoting rhizobacteria (PGPR) are beneficial soil microbes that can stimulate plant growth or increase plant tolerance to stresses. Some PGPR strains release volatile organic compounds (VOCs) that can directly and/or indirectly benefit the plants by increasing plant biomass, disease resistance, and abiotic stress tolerance. This chapter provides an overview of bacterial VOC-induced enhancement of plant tolerance to high salinity and drought stress, as well as of bacterial VOC-mediated improvement of sulfur and iron nutrition in plants.

Keywords Plant growth-promoting rhizobacteria (PGPR) · Volatile organic compounds (VOCs) · Abiotic stress · Salinity · Drought · Sulfur · Iron

7.1 Introduction

Plants in nature are closely associated with a large number of soil microbes, most of which being bacteria. Plant-associated soil microbes either live within the root tissues or in the rhizosphere, which is a thin layer of soil adhering to the roots (Bulgarelli et al. 2013, 2015). Plants release a variety of organic compounds into the

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soil through roots, via a process known as rhizodeposition (Nguyen 2009). The root exudates in the rhizosphere create a nutrient-rich environment for soil bacteria (Bulgarelli et al. 2013, 2015). On the other hand, while some rhizobacteria have no observable effects on plant growth or fitness, a plethora of other rhizobacteria can affect plants either negatively or positively by releasing certain bacteria-derived compounds (Vaishnav et al. 2017a, b). Among the rhizobacteria, plant growth-promoting rhizobacteria (PGPR) are beneficial bacteria that have been successfully used in agriculture to increase seedling emergence, plant weight, crop yield, and disease resistance (Beneduzi et al. 2012; Kashyap et al. 2017). These beneficial soil microbes promote plant growth and improve disease resistance through production of one or multiple bacterial factors including phytohormones such as auxin and cytokinin, the enzyme ACC (1-aminocyclopropane-1-carboxylate) deaminase that reduces plant ethylene levels, and siderophores that facilitate root uptake of metal nutrients (Glick 1999; Timmusk and Wagner 1999; Vaishnav et al. 2017a, b). In addition to these non-volatile compounds, some PGPR strains also release volatile organic compounds (VOCs) capable of affecting plant physiology under normal and stress conditions (Paré et al. 2011; Farag et al. 2013; Liu and Zhang 2015).

Since the first discovery of VOC-mediated bacteria regulation of plant growth (Ryu et al. 2003), bacteria VOC emission has been recognized as an important aspect of plant–microbe interactions, in addition to those interactions that are based on direct contact between the bacteria and the plant (Ryu et al. 2003; Wenke et al. 2010; Blom et al. 2011; Bitas et al. 2013; Farag et al. 2013). Bacteria can produce diverse volatile compounds (Schulz and Dickschat 2007), including both organic and inorganic (e.g., hydrogen cyanide or carbon dioxide) components. Although the identity and abundance of individual VOC components vary among different species, bacterial VOCs commonly can be categorized into the compounds including short-chain aliphatic aldehydes, esters, alcohols, organic acids, ethers, ketones, sulfur compounds, and hydrocarbons (Farag et al. 2006; Vaishnav et al. 2017a). Some VOCs have been shown to be toxic while some others can be beneficial to plants (Rudrappa et al. 2010; Blom et al. 2011; Ryu et al. 2003; Farag et al. 2006). An increasing interest concerning the biological function of bacterial volatiles has been documented and has clearly demonstrated that certain bacteria VOCs can increase plant biomass production and enhance plant resistance to biotic and abiotic environmental stresses (Ryu et al. 2003; Zhang et al. 2007, 2008a, 2009; Choi et al. 2014; Liu and Zhang 2015; Vaishnav et al. 2015; Sharifi and Ryu 2018). In this chapter, we focus on the important roles of bacterial VOCs on plant abiotic stress tolerance (Fig. 7.1).

7.2 Enhancement of Salt Tolerance

High salinity is one of the most adverse environmental factors that severely limits crop productivity, because excessive sodium (Na^+) in soil causes osmotic stress and ionic toxicity to plant cells (Zhu 2001; Rahnama et al. 2010; James et al. 2011;

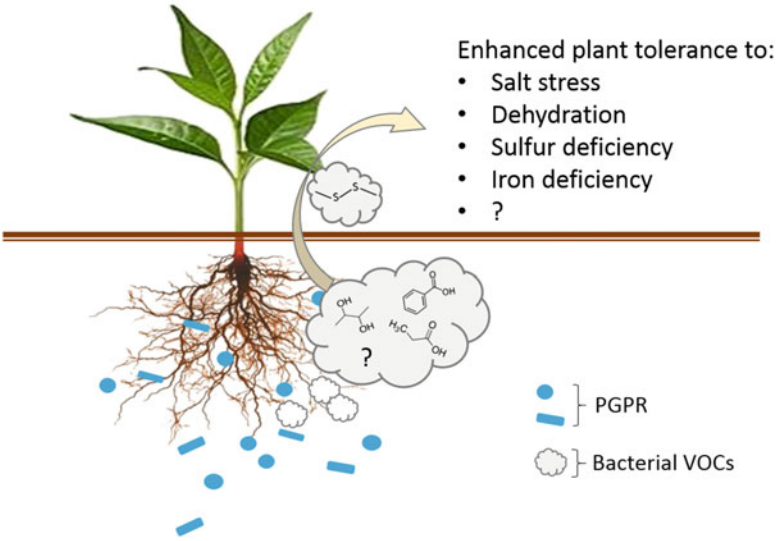

Fig. 7.1 A model of the enhanced plant stress tolerance that is triggered by bacterial volatiles. *PGPR* plant growth-promoting rhizobacteria, *VOCs* volatile organic compounds. Question marks indicate either unknown bioactive VOC components or yet-to-be-explored VOC effects. Also see Table 7.1 for detailed information

Shrivastava and Kumar 2015). Plants grown in saline conditions can minimize Na^+ toxicity by restricting its root uptake and the subsequent loading into xylem, as well as by promoting the shoot-to-root Na^+ recirculation and the Na^+ extrusion from root cells (Tester and Davenport 2003; Munns and Tester 2008; Zhang et al. 2008a; Kronzucker and Britto 2011; Zhang and Shi 2013). Plant tolerance to salt stress can be enhanced by some PGPR strains through bacterial VOC emissions (Zhang et al. 2008a; Vaishnav et al. 2015; Ledger et al. 2016; Bhattacharyya and Lee 2017).

Arabidopsis exposed to the VOCs released from *Bacillus amyloliquefaciens* GB03 acquired enhanced salt tolerance, as shown by the greater biomass production and the lower Na^+ accumulation compared to plants without VOC exposure (Zhang et al. 2008a; Choi et al. 2014). The enhanced plant salt tolerance probably involves HKT1-mediated Na^+ recirculation and SOS3-mediated Na^+ exudation. *Arabidopsis* HKT1 is a xylem parenchyma-expressed Na^+ transporter, which is responsible for Na^+ exclusion from leaves by removing Na^+ from the xylem sap (Sunarpi et al. 2005; Horie et al. 2009; Moller et al. 2009). In salt-stressed *Arabidopsis* plants, GB03 VOCs induced *HKT1* gene expression in shoots and concomitantly reduced shoot Na^+ accumulation; meanwhile, the *hkt1* null mutant accumulated high levels of Na^+ in shoots and was sensitive to salt stress regardless of the presence of GB03 VOCs (Zhang et al. 2008a). These results suggested that GB03 VOC-enhanced plant salt tolerance was mediated through HKT1-dependent shoot-to-root Na^+ recirculation. When stressed by salinity, wild type *Arabidopsis* exposed to GB03 VOCs showed reduced plant Na^+ levels by approximately 50% compared to plants without VOC

treatment; by contrast, in the *Arabidopsis salt overly sensitive 3* (*sos3*) mutant, VOCs triggered only 15% reduction in plant Na^+ levels (Zhang et al. 2008a). SOS3 is required for post-transcriptional activation of the H^+/Na^+ antiporter SOS1, which controls root Na^+ exudation and long-distance Na^+ transport in plants (Shi et al. 2000). Therefore, in addition to HKT1, SOS3 also plays a critical role in mediating GB03 VOC-triggered plant tolerance to salt stress, possibly through its function in regulating root Na^+ exudation. GB03 VOCs contain acidic components and cause rhizosphere acidification (Farag et al. 2006; Zhang et al. 2009), which may result in a proton gradient that facilitates the SOS1-mediated export of Na^+ from roots. These observations with GB03 collectively suggest that plant salt tolerance can be enhanced by bacterial VOCs through integrated regulation of Na^+ homeostasis *in planta*.

Besides modulation of plant Na^+ homeostasis, bacteria VOCs may affect plant accumulation of osmoprotectants including secondary metabolites and stress-responsive proteins. *Pseudomonas simiae* strain AU produces VOCs that were shown to increase soybean tolerance to salt stress (Vaishnav et al. 2015). The volatiles were effective in lowering Na^+ levels and simultaneously increasing accumulation of the osmoprotectant proline in roots. VOCs from *P. simiae* AU also induced the production of several proteins, such as vegetative storage proteins, which are known to maintain plant growth under stress conditions (Vaishnav et al. 2015). *P. simiae* VOCs contain 4-nitroguaiacol and quinolone (Vaishnav et al. 2016). These two volatile compounds, in combination with sodium nitroprusside that is a nitric oxide donor, induced antioxidative enzymes and nitrate reductase gene expression, resulting in promoted germination rates of soybean seeds under salt stress (Vaishnav et al. 2016).

Bacterial regulation of plant hormone signaling pathways can also mediate VOC-enhanced plant tolerance to salt stress. VOCs from *Alcaligenes faecalis* strain JBCS1294 increased *Arabidopsis* tolerance to salt stress by inducing genes involved in auxin and gibberellin signaling pathways (Bhattacharyya et al. 2015). Consistently, JBCS1294 VOCs induced salt tolerance neither in wild type plants treated with auxin or gibberellin inhibitors nor in the *Arabidopsis* mutants *eir1* and *gai-1*, which are defective in the signaling pathways of auxin and gibberellin, respectively. In contrast, the phytohormones cytokinin, brassinosteroid, and ethylene played negative roles in mediating the beneficial effects caused by JBCS1294 VOCs, as shown by genetic disruption or chemical inhibition of the corresponding signaling pathways (Bhattacharyya et al. 2015). Bhattacharyya and Lee (2017) later examined individual components of JBCS1294 VOCs and found that plant salt tolerance could be induced by either butyric acid, propionic acid, or benzoic acid. Interestingly, compared to the individual VOC components, an optimized combination of the three volatile compounds displayed stronger capacity in enhancing plant stress tolerance to salinity, although the same VOC cocktail failed to induce growth promotion in plants without salt stress (Bhattacharyya and Lee 2017). Similar to natural VOCs from JBCS1294, the volatile cocktail failed to increase plant tolerance to salt stress in *eir1* and in wild type plants treated with auxin or gibberellin inhibitors. Intriguingly, *Arabidopsis* plants exposed to GB03 VOCs also displayed altered auxin

homeostasis (Zhang et al. 2007), indicating a potential link between VOC-mediated modulation of plant salt tolerance and of auxin homeostasis. It is noteworthy that VOCs from some fungal species have also been reported to induce plant tolerance to salt stress in an auxin-dependent manner, as indicated by a failure of fungal VOC-induced increases in leaf surface area and lateral root density in the auxin signaling mutants *aux1-7, tir1-1,* and *axr1-3* (Li and Kang 2018).

Similar to the combination of butyric acid, propionic acid, and benzoic acid, another synthetic mixture of bacterial VOC components, including 2-undecanone, 7-hexanol, and 3-methylbutanol, was shown to mimic natural VOCs from *Paraburkholderia phytofirmans* PsJN in promoting *Arabidopsis* growth under both salt-stressed and non-stressed conditions (Ledger et al. 2016). In addition, it was shown that early exposure to *P. phytofirmans* PsJN VOCs was sufficient to stimulate long-term effects on plant growth promotion in the presence and absence of salinity (Ledger et al. 2016).

7.3 Protection from Dehydration

Under high salinity or drought conditions, plant cells suffer from osmotic stress that leads to water loss. Plants respond to dehydration by elevating cellular levels of osmoprotectants, such as choline and glycine betaine, in order to achieve a balanced water potential across the cell membrane and thereby prevention of water loss (Rhodes and Hanson 1993; Yancey 1994).

Arabidopsis treated with VOCs emitted by *B. amyloliquefaciens* GB03 showed increased tolerance to dehydration caused by mannitol supplemented to the growth medium; meanwhile, root inoculation of GB03 enhanced *Arabidopsis* tolerance to drought stress (Zhang et al. 2010). GB03 VOCs induced gene expression of *PEAMT*, which encodes an enzyme critical for choline and glycine betaine biosynthesis in *Arabidopsis*. GB03 VOCs increased the accumulation levels of choline and glycine betaine in osmotic-stressed plants, but failed to enhance plant dehydration tolerance in *PEAMT* defective mutant plants, consistent with a key role of PEAMT in mediating GB03 VOC-induced plant tolerance to dehydration (Zhang et al. 2010). Under osmotic stress conditions, GB03 VOC-treated plants and the untreated plants showed similarly elevated levels of abscisic acid (ABA), which is a stress-responsive phytohormone crucial for plant abiotic stress tolerance (Zhang et al. 2010). This pattern indicates that ABA mediates basal, but not VOC-triggered enhancement of stress tolerance in plants. In some other studies, PGPR-induced plant drought tolerance was not attributed to ABA either, because plants with PGPR treatments displayed lower ABA levels than the untreated plants (Cho et al. 2008; Kang et al. 2014). By contrast, salicylic acid (SA) seems to be critical for plant drought tolerance induced by *Pseudomonas chlororaphis* strain O6 and by 2,3-butanediol, which is a component of the VOCs emitted by *P. chlororaphis* O6 (Cho et al. 2008). This opinion was supported by the observations that drought-stressed plants with exposure to either O6 or 2,3-butandiol displayed higher SA levels compared to their

control counterparts, and that the enhancement of plant drought tolerance was impaired in *Arabidopsis* mutant defective in SA signaling. At physiological level, the plant drought tolerance induced by O6 or 2,3-butanediol was correlated with increased stomatal closure (Cho et al. 2008). Consistently, later studies showed that 2,3-butanediol induced plant production of nitric oxide (NO), which is an important signaling molecule for stress-induced stomatal closure (Cho et al. 2013; Li et al. 2017). Similarly, 2,3-butanediol and acetoin released from *B. amyloliquefaciens* FZB42 activated SA and ABA signaling pathways and stimulated accumulation of hydrogen peroxide and NO, resulting in stomatal closure in Arabidopsis and *Nicotiana benthamiana* (Wu et al. 2018). Notably, Wu et al. (2018) also showed that adding these two bacterial VOCs directly to the soil was more effective in reducing stomatal apertures, compared to the volatilization treatment by setting the volatile compounds and the pot with plants next to each other within a glass container. Application of an inhibitor of nitrate reductase or an inhibitor of nitric oxide synthase decreased NO production as well as plant drought tolerance induced by 2,3-butanediol (Cho et al. 2013); this observation further supported an important role of NO in mediating 2,3-butanediol-induced plant drought tolerance. The compound 2,3-butanediol is also a component of VOCs from *B. amyloliquefaciens* GB03 (Farag et al. 2006), and thus it probably contributes to GB03 VOC-induced plant tolerance to dehydration, although it is unknown whether 2,3-butanediol induces *PEAMT* gene expression as the natural blend of GB03 VOCs does.

Some PGPR strains produce biofilms that are mainly composed of exopolysaccharides. Biofilm production can be induced by certain bacterial VOCs such as acetic acid (Chen et al. 2015); meanwhile, exopolysaccharides improve soil aggregation and maintain soil moisture in the rhizosphere and thus can help plants survive under water deficit conditions (Amellal et al. 1998; Niu et al. 2018). For instance, exopolysaccharide-producing PGPR strains, such as *Pseudomonas aeruginosa* Pa2 and *Pantoea agglomerans* NAS206, have been shown to increase drought resistance in plants (Naseem and Bano 2014). Therefore, VOC-induced plant dehydration tolerance may be achieved indirectly through stimulation of exopolysaccharide secretion, in addition to directly through affecting biological processes in plants.

7.4 Augmentation of Sulfur Acquisition

Sulfur is a macronutrient required for plants throughout the whole life cycle. As an essential element in many pivotal biomolecules, sulfur is important for plant development and for plant resistance to environmental stress (Aziz et al. 2016). Sulfur deficiency causes photosynthesis repression and disruption of primary metabolism (Burke et al. 1986; Gilbert et al. 1997; Liu and Zhang 2015). Plant acquisition of sulfur has been shown to be augmented by VOCs from *B. amyloliquefaciens* GB03 and *Bacillus* sp. B55 through different mechanisms.

Arabidopsis exposed to GB03 VOCs displayed transcriptional up-regulation of sulfur assimilation, as evidenced by VOC induction of the gene families encoding ATP sulfurlyase (ATPS), adenosine 5′-phosphosulfate reductase (APR), and adenosine 5′-phosphosulfate kinase (APK) (Aziz et al. 2016). Monitoring of sulfur uptake and translocation with radio-labeled sulfate ($^{35}SO_4^{2-}$) revealed that VOCs enhanced total sulfur uptake per plant within 30 min. Consistently, sulfur accumulation levels were increased by GB03 VOCs. The enhanced sulfur acquisition apparently resulted in augmented sulfur utilization. For instance, levels of the amino acid cysteine, which is a precursor of many organic sulfur metabolites, were increased by more than 90% in plants with 2 weeks of VOC exposure compared to the control plants. In addition, *Arabidopsis* genes responsible for the production of sulfur-rich aliphatic and indolic glucosinolates were also induced by GB03 VOCs, consistent with the elevated levels of glucosinolates that protected plants from the herbivore beet armyworm (Aziz et al. 2016).

Unlike GB03 VOCs that affect root uptake of SO_4^{2-}, VOCs from *Bacillus* sp. B55 augmented plant sulfur acquisition in the air (Meldau et al. 2013). With radio-labeled sulfur ^{35}S supplemented into the bacteria growth medium, B55-emitted VOCs was shown to transmit sulfur to *Nicotiana attenuata* plants. B55 VOCs contain at least two sulfur-containing compounds, dimethyl disulfide (DMDS) as a major component and *S*-methyl pentanethioate present at trace amounts. Plant growth retardation caused by sulfur starvation was rescued by either the natural VOC blends or synthetic DMDS, with the latter showing higher capacity than the former in rescuing the plant stress symptoms. Therefore, sulfur nutrition present in B55 VOCs was attributed mainly to DMDS (Meldau et al. 2013). Sulfur in SO_4^{2-} is in an oxidative state that needs an energy-consuming reduction process for biological assimilation (Takahashi et al. 2011); by contrast, sulfur in DMDS is in a chemically reduced state. This difference seems to make DMDS superior to SO_4^{2-} by providing energy-saving sulfur nutrition to plants. Consistently, sulfur assimilation as well as methionine biosynthesis and recycling in plants were transcriptionally repressed by DMDS, indicating a decreased demand for SO_4^{2-} reduction (Meldau et al. 2013). DMDS is commonly detected in microbial VOCs; meanwhile, VOCs from microbes other than B55 can contain high levels of other sulfur-containing VOCs such as dimethyl sulfide and dimethyl trisulfide (Kanchiswamy et al. 2015). Thus, VOC-mediated sulfur assimilation might be a common event during plant–microbe interactions.

7.5 Improvement of Iron Homeostasis

Iron is a micronutrient that participates in electron transfer reactions via the transition between ferrous iron (Fe^{2+}) and ferric iron (Fe^{3+}). Although iron element is highly abundant in the earth's crust, it is mostly present as insoluble oxyhydroxide polymers that are not readily taken up by plants (Guerinot and Yi 1994). Iron deficiency disrupts photosynthetic apparatus with easily observable leaf chlorosis symptoms

(Zhang et al. 2009). In order to increase iron mobility in the rhizosphere, graminaceous monocots secrete siderophores to chelate Fe^{3+} before its root uptake, while non-graminaceous monocots and dicots deploy a combined strategy including rhizosphere acidification, reduction of Fe^{3+} to Fe^{2+} by plasma membrane ferric reductase, and transporter-mediated Fe^{2+} import into roots (Curie and Briat 2003).

Bacteria VOCs do not contain any known siderophores; however, acidic compounds are common VOC components (Audrain et al. 2015) and can acidify the rhizosphere. For example, VOCs emitted from *B. amyloliquefaciens* GB03 have been shown to directly cause rhizosphere acidification (Zhang et al. 2009). GB03 VOCs also stimulated *Arabidopsis* root proton exudation, making further contribution to rhizosphere acidification (Zhang et al. 2009). Similarly, VOCs from *Sinorhizobium meliloti* caused rhizosphere acidification by induction of root proton exudation in *Medicago truncatula* (del Carmen Orozco-Mosqueda et al. 2013). Consistently, root ferric iron reductase activities were increased and iron uptake was enhanced in *Arabidopsis* exposed to GB03 VOCs and in *M. truncatula* exposed to *S. meliloti* VOCs. *Arabidopsis* treated with GB03 VOCs also displayed gene induction of FIT1, a transcription activator controlling gene expression of the root Fe^{3+} reductase FRO2 and the Fe^{2+} transporter IRT1. Concomitant with the up-regulation of *FIT1*, transcriptional activation of *FRO2* and *IRT1* was observed in wild type *Arabidopsis* exposed to GB03 VOCs, and was abolished in the *fit1* null mutant. Plant exposure to GB03 VOCs induced leaf cell expansion and photosynthesis augmentation (Zhang et al. 2007, 2008b). Given the importance of iron and sulfur to photosynthesis, it is possible that VOC-enhanced nutrient uptake is part of its coordinated regulation of plant biological processes. As a result of the fortified iron nutrition, increases in chlorophyll contents were observed in *Arabidopsis* exposed to GB03 VOCs and in *M. truncatula* exposed to *S. meliloti* VOCs. Consistent with VOC-mediated enhancement of iron uptake, direct inoculation of GB03 to cassava resulted in elevated iron accumulation (Freitas et al. 2015).

Plants that rely on siderophores for Fe^{3+} uptake also possess FRO genes. Strong induction of the leaf-specific *FRO1* gene was observed in *Sorghum bicolor* treated with VOCs emitted from *Arthrobacter agilis* UMCV2; concomitantly, the plants exhibited increases in chlorophyll content and biomass production (Castulo-Rubio et al. 2015). Dimethyl hexadecilamine (DMHDA), a component of *A. agilis* UMCV2 VOCs, was shown to mimic the natural VOC blends in triggering *FRO1* gene expression and plant growth promotion.

7.6 Future Perspectives

VOC emission is a common property of a wide variety of soil microorganisms. Although to date only a small number of PGPR strains have been reported to enhance plant tolerance to abiotic stresses, research on this topic has demonstrated the important role of VOCs (Table 7.1). The increasing interests in microbial VOCs should lead to identification of more VOCs that can enhance plant abiotic stress

Table 7.1 A list of bacterial volatile organic compounds that affect plant abiotic stress tolerance

PGPR effects on plants	PGPR species (strain)	Bacterial VOCs	In planta mediators	Plant species	References
Salt stress tolerance	*Bacillus amyloliquefaciens* (GB03)	Entire VOC blend	HKT1; SOS3	*Arabidopsis*	Zhang et al. (2008a)
Salt stress tolerance	*Pseudomonas simiae* (AU)	4-Nitroguaiacol; quinolone	Proline; antioxidative enzymes; nitrate reductase	Soybean	Vaishnav et al. (2015, 2016)
Salt stress tolerance	*Alcaligenes faecalis* (JBCS1294)	Butyric acid; propionic acid; benzoic acid	Auxin; gibberellin	*Arabidopsis*	Bhattacharyya et al. (2015) and Bhattacharyya and Lee (2017)
Salt stress tolerance	*Paraburkholderia phytofirmans* (PsJN)	2-Undecanone; 7-hexanol; 3-methylbutanol	Unknown	*Arabidopsis*	Ledger et al. (2016)
Osmotic stress tolerance	*Bacillus amyloliquefaciens* (GB03)	Entire VOC blend	PEAMT; glycine betaine; choline	*Arabidopsis*	Zhang et al. (2010)
Drought stress tolerance	*Pseudomonas chlororaphis* (O6)	2,3-Butanediol	Salicylic acid; nitric oxide	*Arabidopsis*	Cho et al. (2008, 2013)
Stomatal closure	*Bacillus amyloliquefaciens* (FZB42)	Acetoin; 2,3-butanediol	Salicylic acid; abscisic acid	*Arabidopsis*; *Nicotiana benthamiana*	Wu et al. (2018)
Improved sulfur acquisition	*Bacillus amyloliquefaciens* (GB03)	Entire VOC blend	ATP sulfurlyase; APRs; APKs	*Arabidopsis*	Aziz et al. 2016
Improved sulfur acquisition	*Bacillus* sp. (B55)	Dimethyl disulfide	Sulfur assimilation pathway	*Nicotiana attenuata*	Meldau et al. (2013)
Improved iron acquisition	*Bacillus amyloliquefaciens* (GB03)	Entire VOC blend; acidic components	FIT1; IRT1; FRO2; root proton exudation	*Arabidopsis*	Zhang et al. (2009)

(continued)

Table 7.1 (continued)

PGPR effects on plants	PGPR species (strain)	Bacterial VOCs	In planta mediators	Plant species	References
Improved iron acquisition	*Sinorhizobium meliloti*	Entire VOC blend	Root proton exudation	*Medicago truncatula*	del Carmen Orozco-Mosqueda et al. (2013)
Improved iron acquisition	*Arthrobacter agilis* (UMCV2)	Dimethyl hexadecilamine	FRO1	*Sorghum bicolor*	Castulo-Rubio et al. (2015)

tolerance. Meanwhile, potential complexities of VOC effects, such as dosage-dependent effectiveness, need to be taken into consideration (Liu and Zhang 2015). Importantly, key questions to be addressed include (1) what is the bioactive VOC component (or combination) that is responsible for the function of the natural VOC blends; (2) how are the bioactive VOCs perceived or utilized in the plants; and (3) how do the VOCs integrally modulate different biological processes in plants to achieve multiple beneficial effects. In addition to addressing interesting scientific questions, an equally important issue is to develop effective methods to apply natural or synthetic VOCs in agriculture for improved crop stress tolerance.

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Chapter 8
Integration of Bacterial Volatile Organic Compounds with Plant Health

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Abstract The integration of a complex of pathways involving growth promotion and stress tolerance in response to bacterial volatile organic compounds (VOCs) is remarkable in enhancing plant health. VOCs are emitted from microbes associated with the plant. In this chapter, we focus on the mechanisms involving the array of structurally diverse VOCs that are active in maintaining a balance for the plant under abiotic and biotic stress. Certain VOCs boost plant growth, act as direct deterrents of pests, or are activators of systemic plant defense mechanisms. The responses to the VOCs involved in plant health engage many different trophic levels and advantageously can occur at distance from the original signal. Thus, VOC sensing has a holistic aspect for plant welfare.

Keywords Fe metabolism · Induced systemic tolerance · Integrated networking · Plant growth stimulation · Recruited microbiome

8.1 Background

The sessile plant defends itself against stress using physical and chemical strategies. The use of VOCs provides a mechanism to rapidly signal in three dimensions over long and short distances, permitting responses in the same plant, other nearby plants, and in organisms at the interkingdom levels (Audrain et al. 2015; Tyc et al. 2017). Effects of the VOCs operate both above- and below-ground as discussed in previous reviews (Effmert et al. 2012; Schenkel et al. 2015). The headspace around a plant is filled with a mixture of VOCs emitted by many life forms, including insects, bacteria, and fungi, and the tissues of the plant, with both shoot and root emissions

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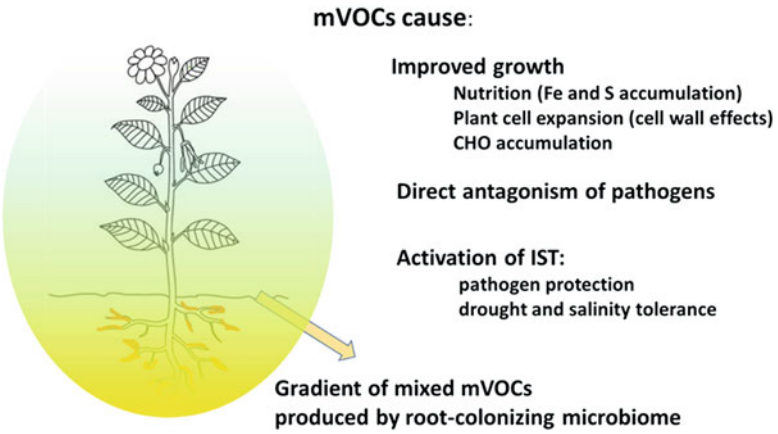

Fig. 8.1 Illustration of potential cloud of mVOCs around a plant with roots colonized by beneficial bacteria (orange overlay on roots). The mVOCs would be a mix of different VOCs that have an impact on plant metabolism. Notable are increased growth and improved defense for both direct and plant - activated mechanisms against biotic and abiotic stress

being involved. The VOC vocabulary from plants alone is over 30,000 chemicals, with an array of structures and different extents of volatility and water solubility. The bouquet includes hydrocarbons, alkanes, and alkenes and their oxidized states, the aldehydes, alcohols, esters, ketones, and acids; N-containing structures, complex ring structures including furans and terpenoids, and S-derivatives (Effmert et al. 2012; Hammerbacher et al. 2019; Schenkel et al. 2015; Tyc et al. 2017). Lemfack et al. (2014) established a microbial volatile organic (mVOC) database with 1093 compounds catalogued. Analysis published in 2015 found that of the 39 VOCs identified from roots of maize, *Arabidopsis*, and fava bean, 28 are also classified as microbial volatiles (Schenkel et al. 2015). Genetic analysis of their synthetic pathways might reveal whether these result from genes shared between microbes and plants over evolutionary history. Alternatively, they may have been synthesized from microbes associated with the plant rather than the plant itself.

The structures and sources of VOCs relevant to plant health are discussed in many recent reviews (Bitas et al. 2013; Fincheira and Quiroz 2018; Kanchiswamy et al. 2015; Piechulla et al. 2017; Sharifi and Ryu 2018; Weisskopf et al. 2016). The VOCs have diverse effects on plant metabolism. In addressing VOCs and plant health, the responses include a boost in growth through mechanisms that include changes in nutrition and plant growth regulator responses, direct inhibition in growth of potential pathogens (nematodes, insects, and microbes), recruitment of beneficial organisms that will affect a pathogenic challenge directly, and induced resilience in the plant to both biotic and abiotic stress.

This chapter introduces examples of different mechanisms involving VOC-induced plant responses, or changes that are of consequence to the plant's health (e.g., reduced pathogen pressure). The mechanisms discussed in the following sections and summarized in Fig. 8.1 are:

1. Microbial VOCs and plant growth stimulation,
2. Microbial VOCs and direct inhibition of challenge,
3. Microbial VOCs and activation of induced systemic tolerance, and
4. Successful integration of plant responses for VOC-induced health.

8.2 Microbial VOCs and Plant Growth Stimulation

Numerous microbes promote plant growth and root-colonizing bacteria with this property are termed plant growth-promoting rhizobacteria (Kloepper and Schroth 1981). However, the knowledge base is very much at the level of an incomplete puzzle with the interests of different research groups providing pieces that lock together; however, there are pieces that are still missing. The plant participates in this process by providing nutrients to the plant growth-promoting rhizobacteria as root exudate components (Hu et al. 2018).

The most frequent assay for microbial VOCs is their identification from headspaces when organisms are grown on artificial medium; however, such media may contain compounds that are metabolized by the microbes but may not be present in the natural environment. The in vitro growth also lacks other features conditioning microbial metabolism in planta, such as oxygen and carbon dioxide levels that will affect the onset of fermentation pathways. Additionally, soil factors including pH and composition (especially that of the clays) will alter the bioavailability of a volatile array, for instance, by differentially absorbing the VOCs (Asensio et al. 2008; Stotzky et al. 1976). However, the in vitro assays demonstrate the potential for VOC responses to be produced and excite responses in the targets.

Till recently, studies have been dominated by VOCs from *Bacillus* isolates, but these have now extended to VOCs from many bacterial genera, as well as plant-associated fungi, including *Fusarium* and *Trichoderma* species (Fincheira and Quiroz 2018). Because the VOC composition from a single microbe is affected by the growth medium (Blom et al. 2011), there are reports of differences in the array of compounds that are generated. Indeed, changing the growth medium for *Bacillus mojavensis* RRC101 shifts the VOCs from being plant growth-promoting to inhibitory (Rath et al. 2018). The effects of a single identified compound when present alone versus in a cocktail of many compounds, many of which could lack characterization, are not fully understood.

Increased plant mass caused by the VOCs is manifest in ways that display the innate plasticity in plant growth. Increased leaf surface area and enhanced lateral root formation are two processes induced by VOCs (Ditengou et al. 2015; Ryu et al. 2003). For instance, Park et al. (2015) found VOCs from *Pseudomonas fluorescens* SS101 promoted plant growth in vitro and in soil, with 13-tetradecadien-1-ol, 2-butanone, and 2-methyl-*n*-1-tridecene being the most active of the 11 VOCs detected. Plant growth promotion manifested as increased leaf surface area is also associated with the microbial fermentation products, acetoin and 2S,3R-butanediol. Although initially documented as VOCs from *Bacillus* isolates, many other microbes also generate these products (Han et al. 2006; Rath et al. 2018; Ryu et al. 2003).

Several mechanisms are identified and illustrate the extent of changes induced in the plants' metabolism to achieve growth promotion by mVOCs. Modified cytokinin levels are implicated in the promotion of growth and flowering by VOCs from the fungus, *Alternaria alternata* (Sanchez-Lopez et al. 2016). These studies confirm the accumulations of starch and sucrose observed previously in leaves after mVOC treatments (Ezquer et al. 2010). Early studies illustrate connections between Fe nutrition, photosynthetic efficacy, and plant growth regulators in the responses of *Arabidopsis* to VOCs from *Bacillus subtilis* GB03. Zhang et al. (2007) correlate leaf surface area effects with changes in auxin root/shoot partitioning and enhanced expression of plant cell wall-loosening enzymes. Reduced ABA levels and improved photosynthesis efficacy occur in part owing to higher chlorophyll levels as well as enhanced Fe loading (Zhang et al. 2008). Greater bioavailability of Fe involves several features: increased proton release from roots, and expression of a regulator, FIT1, that conditions expression of genes encoding the Fe transporter IRT1 and the ferric reductase FRO2 for *Arabidopsis* (Zhang et al. 2010). This process is independent of Fe deficit in the plant when they are triggered by the VOCs from GB03. Similar enhanced Fe acquisition in *Arabidopsis*, independent of Fe deficit, occurs with VOCs from a pseudomonad (Zamioudis et al. 2015). In *Arabidopsis* exposed to the VOCs from *Bacillus amyloliquefaciens,* higher Fe availability is coupled with increased sulfate transporters which are proposed to explain elevated selenium loading into the plant (Wang et al. 2017).

Increased growth is also associated with the higher availability of sulfur (S) derived from plant metabolism of the VOC, dimethyl disulfide, a common VOC from microbes when grown on a peptide-rich medium (Meldau et al. 2013). Enhanced S metabolism in planta occurs with VOCs from *B. subtilis* GB03; the VOC-induced increases in glucosinolates are tied to resistance to herbivory (Aziz et al. 2016). Work with H_2S, a microbial inorganic volatile, shows a suite of changes including improved photosynthesis efficacy and altered thiol redox status (Chen et al. 2011). Indeed, GYY-4137, a formulation for slow release of H_2S, results in improved plant growth such that it is considered for commercial use (Carter et al. 2018). Studies by Aroca et al. (2017) indicate that persulfidation of cysteines is a trigger for altered metabolism in *Arabidopsis* exposed to H_2S. In response, there are major changes in primary metabolism, i.e., glycolysis, the TCA cycle, and photosynthesis. At high doses of H_2S, inhibition of root growth is observed (Zhang et al. 2017). This morphological change correlates with NO and H_2O_2 signaling and impaired auxin distribution in the root (Zhang et al. 2017).

Because of the complexity of the potential VOC array from each microbe depending on available nutrition, uncovering the active players in changing plant growth and the nature of the mechanisms involved is complex and currently exists largely at the phenotypic level rather than at the molecular level. When in the field, another layer of complexity arises from the association of the plant with the many microbes present in its microbiome with differences between leaf and root surfaces and the endophytic populations.

8.3 Microbial VOCs and Direct Inhibition of Pathogen Growth

VOCs are among the suite of antimicrobial compounds with biocontrol potential produced by microbes (Fig. 8.1). These observations present the possibility that the VOCs can be formulated as biofumigants (Zhang et al. 2019). This possibility was realized for postharvest control of black rot of sweet potato by the VOCs 3-methyl butanol, phenylethyl alcohol, and 2-methyl butanol, produced by an isolate of *Pseudomonas chlororaphis* subsp. *aureofaciens.* These VOCs directly inhibit conidial germination and mycelial growth of the fungal pathogen causing black rot (Zhang et al. 2019). The VOCs of another pseudomonad, *P. fluorescens* WR-1, inhibit replication of the bacterium, *Ralstonia solanacearum,* that causes tomato wilt (Raza et al. 2016).

A root endophytic *P. putida*, isolate BP25, successfully controls infections of pepper by the microbial pathogen, *Phytophthora capsici*, as well as the burrowing nematode, *Radopholus similis*; in vitro assays identified 16 bacterial VOCs, and this complex inhibits the growth of both these antagonists (Sheoran et al. 2015). Similarly, pests from three kingdoms (microbes, nematodes, and insects) are growth - inhibited by VOCs from a group of pseudomonad isolates (Popova et al. 2014). More examples for mVOCs that are antagonistic to microbes are reviewed by Bitas et al. (2013).

The mechanisms of how the mVOCs achieve their success await full elucidation. However, in studies with *B. subtilis* GB03, a novel finding for one of the mechanisms involved in direct control of the fungal pathogen, *Botrytis cinerea*, is that the VOCs reduce the efficacy of attachment of the mycelium to the host leaf surface (Sharifi and Ryu 2016). The molecular basis of this change to the surface of the fungal pathogen is not yet resolved and whether similar changes occur with bacterial interactions is not known.

Some of the volatiles are fungistatic rather than fungicidal, as shown by studies with *Trichoderma* isolates; the volatile 6-pentyl-alpha-pyrone was the active volatile tested against the fungal plant pathogen, *Fusarium moniliforme,* in which conidial germination, germ tube elongation, and mycelial growth were each impaired (El-Hasan et al. 2007). Such fungistasis could be the first part of a resistance mechanism, slowing the progress of the pathogen until protective measures from the plant could be activated.

8.4 Microbial VOCs and Activation of Induced Systemic Tolerance

In addition to direct effects on the pathogen, many mVOCs from protectant microbes prime the plants to react faster and with higher efficacy with induced systemic tolerance (IST) when subsequently challenged (Mauch-Mani et al. 2017). Thus,

the tissues and even nearby plants become ready with better defense for a pathogen or insect (Scala et al. 2013). For instance, Sharifi and Ryu (2016), demonstrate this priming is a major process involved in protection against the grey mold necrotroph, *Botrytis cinerea,* by *B. subtilis* GB03, with direct antagonism being of lesser importance. They found that the bacillus VOCs activate expression of both the jasmonic acid (JA) and salicylic acid (SA) defense pathways; upregulation of the marker genes of PDF1.2 in the JA pathway and PRI for the SA pathway occurs in *Arabidopsis* in response to GB03 VOCs (Sharifi and Ryu 2016). Plant protection from microbial pathogens by systemic activation of these two mechanisms is well established and features of the molecular interactions required for each pathway are resolved (Pieterse et al. 2014; Thatcher et al. 2005). The defensin PDF1.2 in *Arabidopsis* is associated with defense against microbial pathogens that cause necrosis. The JA pathway also is effective in defense against insect damage and protection against cold stress (Cofer et al. 2018). Consequently, triggering the defense pathways can have multiple benefits for the plant.

8.4.1 Osmolyte Accumulation and Stomatal Closures: Functional Measures in Stress Tolerance

Liu and Zhang (2015) extend GB03 VOC-induced stress tolerance to include plant protection against drought and salinity. They correlate increased drought tolerance to heightened levels of plant protectant osmolytes, such as glycine betaine, proline, and choline. The osmolytes could aid in maintenance of cell turgor (Bartels and Sunkar 2005; Kim et al. 2012). Other plant metabolites are also involved in osmotic balance: sucrose, trehalose, and raffinose family oligomers, and the polyamines, which themselves have volatile properties (Hoekstra et al. 2001). Plant-generated galactinol and choline play key roles in tolerance against drought stresses when induced by *B. subtilis* GBO3, and the Gram-negative bacterium, *P. chlororaphis* O6 (Cho et al. 2008; Kim et al. 2008; Zhang et al. 2010).

An additional factor involved in drought tolerance is the partial closure of stomates, conserving water retention within leaves, a trait correlated with the presence of the pyruvate fermentation products, acetoin and 2R,3S-butanediol in the VOCs from the *Bacillus* isolate, GB03. This effect could be coupled with other triggers for stomatal closure that are part of the innate immunity response in plants against plant pathogens. The flagellar peptide, flg22, and the lipopolysaccharide component of the outer membranes of Gram-negative cells induce stomatal closure when applied to leaves (Melotto et al. 2006). Indeed drought stress tolerance in plants accompanies observed stomatal closure with treatments of flg22 and lipopolysaccharide under drought or normal growth conditions (Cho et al. 2008).

The observed activation of genes for HKT1, a Na^+ transporter, differentially in shoot and root tissues, plus altered expression of a gene encoding a root H^+/Na^+ antiporter could account for lower Na^+ load in the saline-stressed plants exposed to

B. subtilis GB03 VOCs. These changes for abiotic stress are coincident with the other processes that promote plant growth, including enhanced Fe loading, as discussed in Sect. 8.1. Zamioudis et al. (2015) link the induction of systemic tolerance by uncharacterized VOCs from a pseudomonad, *Pseudomonas simiae* WCS417, with enhanced iron uptake in *Arabidopsis* roots, to increases in a specific transcription factor, MYB72. This response, which occurs in the plant without Fe deficiency, also requires a factor linked with photosynthesis. Follow-up studies (Stringlis et al. 2018) show that MYB72 is involved in root secretion of the iron chelator, scopoletin, which has direct antmicrobial activity and, thus, has the potential to be involved in biocontrol.

8.4.2 2R,3S-butanediol, a Keystone VOC for IST

2R,3S-butanediol is one of the microbial VOCs for which some knowledge is available of the mechanism responsible for defense pathway activation. Because responses are specific to the stereoisomer (Kong et al. 2018) some level of dimensional recognition is involved but a plant receptor for this mVOC has not been identified. In the Gram-negative, root-colonizing pseudomonad, *P. chlororaphis* O6, deletion of a global regulator GacS eliminates 2R,3S-butanediol synthesis; thus, butanediol synthesis is among the large array of traits that are controlled by the population density-dependent Gac system (Han et al. 2006). It is possible that there is a connection with the lower oxygen supply in high cell density biofilms of *P. chlororaphis* O6 on plant roots with its fermentation of pyruvate to 2R,3S-butanediol. Stomates close in response to 2R,3S-butanediol by mechanisms involving two plant primary signaling molecules, NO and H_2O_2; ABA is not directly involved (Cho et al. 2008). The involvement of both the JA/Et and the SA pathways in 2R,3S-butanediol-activated stomatal closure, as revealed by *Arabidopsis* mutants defective in these pathways (Cho et al. 2013), indicates that this VOC primes the plant for better response to both biotic and abiotic stresses. An interesting consequence of *Arabidopsis* exposure to 2R,3S-butanediol is that the root exudates are modified to become inhibitory to the growth of an isolate of the beneficial fungus *Trichoderma* and a plant pathogen, *Ralstonia solanacearum* (Yi et al. 2016). Such changes in root exudation may be a consequence of the tolerance pathways incited by 2R,3S-butanediol. Yi et al. (2016) also found 2R,3S-butanediol production by *B. subtilis* GB03 promoted its robustness in root colonization. Because 2R,3S-butanediol promotes biofilm formation in *Pseudomonas aeruginosa* (Venkataraman et al. 2014) and *B. subtilis* (Pisithkul et al. 2019), the robustness of the beneficial microbe in planta may be connected to this trait.

8.4.3 *Indole, an Indirect Activator of Plant Defense*

Indole, a volatile metabolite produced from tryptophan by many bacteria (Lee and Lee 2010), affects metabolism in both the plant and the microbes. For instance, indole influences biofilm formation in the potential root colonists, *P. aeruginosa* and *Agrobacterium* (Lee and Lee 2010), and antibiotic resistance in *P. aeruginosa* and *P. fluorescens* (Kim et al. 2017; Lee and Lee 2010). Thus, these traits of importance in plant - microbe associations could be affected when indole is generated from exogenous sources, such as indole-producing bacteria. Indole also is produced rapidly in plants in response to insect regurgitant and wounding (Erb et al. 2015), so that these types of challenges additionally could influence the effects of plant-associated beneficial bacteria. Indole, being a primer for JA-pathway responses, generates IST for plant protection against plant pathogens that cause necrosis. Increased production of JA-isoleucine stimulates plant ABA responses and primes neighboring maize plants for faster defensive responses upon herbivory challenge. Indole elicits a burst of H_2O_2 production with activation of the ROS-responsive MAPK cascade (Shen et al. 2018). This finding correlates with the concept that changes in redox signaling is a key event in the process of priming (Gonzalez-Bosch 2018). In the plant, indole increases production of plant VOCs, the sesquiterpenes, monoterpenes, and homoterpenes. There is evidence that the sesquiterpene, caryophyllene, binds to a TOPLESS (TLP) family protein to act as corepressor of gene transcription in the plant (Nagashima et al. 2018). Other TLPs are already allocated to roles in the regulation of the JA pathway, for instance, to target a complex to the proteasome allowing release of a transcription factor promoting active expression of genes in the JA pathway (Perez and Goossens 2013). These findings illustrate the complexity of a chain of responses, starting with microbially - produced indole influencing responses with other microbes associated with the plant followed by plant responses that result in IST to several stresses.

8.5 Successful Integration of Plant Responses for VOC-Induced Health

The outcome of the findings of the broad range of studies on mVOCs is that their responses play a large role in promoting plant health under stress. What seems remarkable is that the plant manifests an umbrella of potential defenses active for abiotic stress, as well as damage caused by insects and microbial pathogens, without major growth collapse under such altered metabolism. The pathways induced by VOCs correlating with improved growth, such as boosts in photosynthesis and the accumulation of sugars as an energy source, are likely important in maintenance of plant metabolism. Indeed, transcriptome analysis of *Arabidopsis* responding to VOCs from *B. amyloliquefaciens* confirms that genes for plant growth, including modified hormonal effects, cell wall changes as well as plant defense are all

upregulated (Hao et al. 2016). Achieving these goals is likely facilitated by the array of different VOCs that each organism can produce. Each of the VOCs causes a defined spectrum of responses that become integrated to provide the umbrella of coverage for stress and growth observed in the host plant (Fig. 8.1). There is a large output of energy into these VOC-triggered protection schemes in the plant.

The recruitment of metabolites from plant-associated fungi and bacteria for plant health is not surprising. This phenomenon has already been addressed in considering beneficial microbes as probiotics for the plant (Kim and Anderson 2018). The use of VOCs allows rapid access to three-dimensional space, independent of the presence of liquid water and physical contact. Capitalizing on VOCs as signaling molecules, in addition to their role as simple nutrient supplies, allows an ability to change metabolism to rapidly deal with stress in the plant world.

8.6 Conclusion

Plants have coopted the highly variable plasticity of microbial metabolism to boost their own survival probably because plants established into a microbially dominated world. The mVOCs elicit plant defense, directly alter pathogen growth and modulate plant central C metabolism. Prokaryotes are postulated to have arisen about 3.5 Ga with the later development of fungal-like organisms at about 1 Ga. Prior to the existence of land plants, multicellular associations evolved where fungi acted as a matrix and provided minerals from the environment in exchange for the carbon being fixed by photosynthetic microbes. The earliest land plants developed with only minimal soil layers such that mining for minerals and protection from climate and predation was of paramount importance. These needs were likely achieved in part by their association with endophytic fungi like the present-day arbuscular mycorrhizae. This finding is evidenced in the fossil records from the Early Devonian, about 410 Ma (Lambandeira 2006). Similar to the present, these fungi likely aided in providing nutrients and water to the plant but also participated in plant protection through modulation of plant immunity (Strullu-Derrien et al. 2018). Sensing for foe or benefactor is a key evolutionary trait in all organisms.

Currently, we know that bacteria closely associate with mycorrhizal structures (Frey-Klett et al. 2007; Garbaye 1994). Consequently, it is probable that the earliest plants and their fungi were also colonized and influenced by prokaryotes; such associations would not be preserved in the fossil record. Chemosensing exists in bacteria, and its coupling with motility allows for movement towards potential food materials and away from toxins. The bacterial mechanism for detection includes protein modification to alter function, a tool finely tuned to include many variants in eukaryotic cells. Thus, it seems feasible that mechanisms for VOC sensing in plants have evolutionary age. Interestingly, the same Early Devonian fossils that show invasion of the primitive plant by arbuscular mycorrhizal-like fungi also show evidence for arthropod herbivory, boring, and piercing and the insect fossils suggest that winged flight existed (Lambandeira 2006). Phylogenetic analysis confirms the

fossil find. The presence of insects at least by the early Devonian (Misof et al. 2014) suggests that measures to limit damage by insects were needed as land plants were establishing. Later plant fossils indicate that with the evolution of true roots (Kenrick and Strullu-Derrien 2014) an intimate association with mycorrhizal-like fungi was maintained and that these fungi also showed radiation in diversity (Strullu-Derrien et al. 2018). Thus, the current systems of recognition and response to mVOCs likely owe success to the long history of evolution of plant protection mechanisms. The knowledge that we gain on the structure and activity of the VOCs sets the scene for the development of new pest control measures.

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Chapter 9
Volatile Interplay Between Microbes: Friends and Foes

Sébastien Bruisson, Gabriele Berg, Paolina Garbeva, and Laure Weisskopf

Abstract Emission of volatile organic compounds (VOCs) has emerged as important mean of communication between bacteria and other organisms. Most of the knowledge accumulated so far in this field has been obtained with model organisms grown in pure culture. However, in nature, bacteria are part of complex ecosystems and communities encompassing other bacteria, fungal, oomycete, protist, plant, and animal partners. In such communities, bacterial emission of volatiles will be influenced by the surrounding partners and their own volatile emission. This chapter aims at placing bacterial volatile-mediated communication in its global context and summarizing the available literature on how interactions between bacteria and other organisms shape volatile emissions as well as the outcome of biological interactions.

Keywords Volatilome · Inter-kingdom interactions · Microbial communities · Volatile-mediated communication · Induction of volatile emission

9.1 The Microbiome and Its Volatilome

Already in 1988, Whipps et al. defined the microbiome as a characteristic microbial community occupying a reasonably well-defined habitat, which had distinct physiochemical properties (Whipps et al. 1988). The microbiome not only refers to the microorganisms involved but also encompasses their theatre of activity, which results in the formation of specific ecological niches. All living microorganisms

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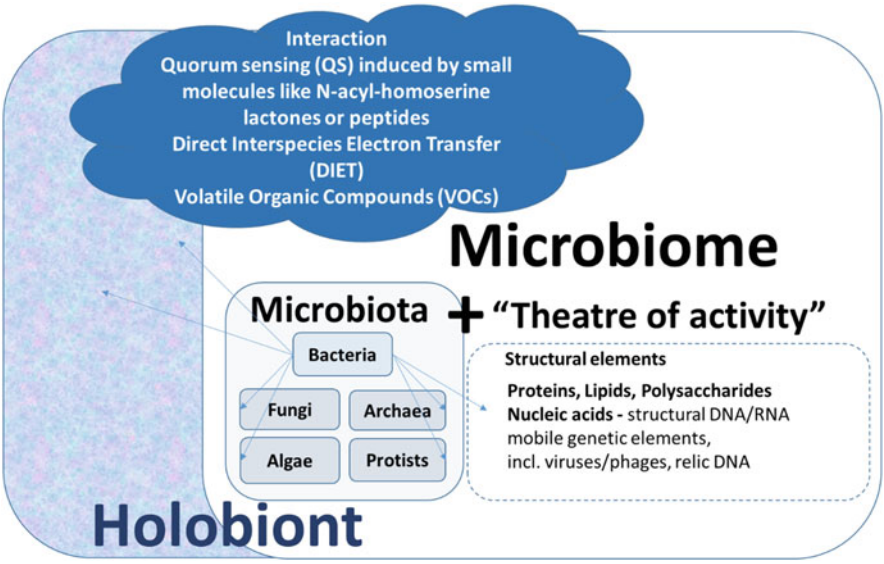

Fig. 9.1 The host–microbiome context of interactions

belonging to different branches of the tree of life, e.g., bacteria, archaea, fungi, algae, and protists form together the microbiota as a part of the microbiome (Marchesi and Ravel 2015). Phages, viruses, plasmids, and mobile genetic elements as well as extracellular DNA derived from dead cells ("relic DNA") are "as theatre of activity" part of the microbiome but not of the microbiota (Fig. 9.1). The microbiome forms a dynamic micro-ecosystem prone to change in time and scale, and is integrated in macro-ecosystems including eukaryotic hosts, where it is crucial for their functioning and health. Therefore, the members of the microbiota intensively interact with each other as well as with their hosts.

The interactive patterns within the microbiota may be positive, negative, or neutral and altogether they have diverse consequences for microbial fitness, population dynamics, and functional capacities within the microbiome (Banerjee et al. 2018). Stability and plasticity of a complex microbial ecosystem depends on these interactions; however, they are so far understudied, especially under natural conditions. Microbial interactions can be an important basis for evolutionary and co-evolutionary dynamics within microbiomes (Gould et al. 2018).

Many microorganisms live in close association with eukaryotic hosts; also they are divided into parasitic, saprotrophic, and neutral interaction based on the impact on their hosts (Lederberg and McCray 2001). Categorizing microbes into parasitic, saprotrophic, and neutral ones may be useful for studies where microbe–host interactions play a central role in mediating host fitness, such as medical studies. However, the interpretation of the pathogenicity data should be taken with caution (Brader et al. 2017). Recent studies on opportunistic pathogens have shown that host–microbe interactions depended not only on the host, but also on the entire

microbiome (Brown et al. 2012; Erlacher et al. 2014). Naturally disease-suppressive soils suggest a similar situation for plants and many opportunistic soil-borne pathogens (Berg et al. 2017; Raaijmakers and Mazzola 2016). Furthermore, in many environmental studies, there may be no specific host available for extended periods of time, making the division into pathogens and beneficials irrelevant. Instead of looking into the interaction of one specific microorganism with its host, one can consider the holistic approach based on the holobiont or super-organism concept. Host–microbe coevolution leads to specific microbiomes associated with plants and animals (Berg and Smalla 2009; Sieber et al. 2019). The theory of the disappearing microbiota suggests that prevalent chronic diseases are caused by the anthropogenic microbiome shifts toward reduced diversity (Blaser 2011).

Secondary metabolites play an essential role in mediating complex interspecies interactions within the microbiome as well as between the microbiome and its host. Quorum sensing (QS) induced by small molecules like *N*-acyl-homoserine lactones or peptides allows bacteria to control cooperative activities and adapt their phenotypes to the biotic environment, resulting, e.g., in cell–cell adhesion or biofilm formation (Bassler 2002). Direct interspecies electron transfer (DIET) is an important mechanism for communication in most anaerobic ecosystems (Lovley 2017). The term "volatilome" contains all volatile metabolites as well as other volatile organic and inorganic compounds that originate from an organism, super-organism, or ecosystem (Bicchi and Maffei 2012). Especially bacteria are well-known producers of volatile organic compounds (VOCs). Fungi are also known to emit a broad range of VOCs (Dickschat 2017); less is known about volatile metabolites of archaeal and protist origin (Schulz-Bohm et al. 2017a). These volatile compounds can act as long-distance messengers for cross-kingdom communication over large areas (Schmidt et al. 2015) (Fig. 9.1). This means that volatile interaction is one of the most important communication skills microorganisms deploy to interact with each other or their host.

The bacterial volatilome encompasses many different types of molecules, many of which have already been identified, while many others remain to be characterized (Audrain et al. 2015a; Tyc et al. 2017a). They can be divided into two main categories, the organic and inorganic compounds. The volatile organic compounds (VOCs) are mostly composed of terpenes, alkanes, nitrogen-containing compounds, ketones, pyrazines, sulfur-containing compounds, and acids. The inorganic volatile compounds are mainly represented by nitric oxide (NO), hydrogen sulfide (H_2S), hydrogen cyanide (HCN), ammonia (NH_3), and carbon dioxide (CO_2).

The composition of a volatilome is not constant; it can vary depending on various parameters. Monitoring of volatiles emitted by bacteria over time has shown that some compounds are produced in a transient manner whereas others, probably related to secondary metabolism, are emitted without interruption from a given time point on, as observed in *Paenibacillus polymyxa* (Rybakova et al. 2017). Both biotic and abiotic components of the bacterial environment have a major impact on the volatilome composition (Blom et al. 2011; de Boer et al. 2019; Raza et al. 2015; Lazazzara et al. 2017). Bacteria are able to sense many changes in their environment, including physico-chemical changes (e.g., pH, temperature) or

changes in the presence of other microorganisms of the same or of different species (Leroux et al. 2015). Thus, they can detect the presence of other bacteria through direct contact, diffusible compound detection (Rybakova et al. 2017; Raza et al. 2015; Leroux et al. 2015; Hibbing et al. 2010), or airborne volatile compound recognition (Audrain et al. 2015a; Westhoff et al. 2017). This ability to sense both types of chemical cues might enable bacteria to detect other organisms located both in closest vicinity and at a greater distance in the microbial world.

Bacteria can also react to changes in their environment by producing volatiles that will have a specific effect on different types of target organisms. Among the most well-known effects of these volatiles are antimicrobial (Rybakova et al. 2017; Kai et al. 2018; Montes Vidal et al. 2017), antifungal (Riclea et al. 2012), and microbiostatic activities (Kanchiswamy et al. 2015; Audrain et al. 2015b; Cornelison et al. 2014; van Agtmaal et al. 2015), along with repellent or chemoattractant properties (Schulz-Bohm et al. 2017a; Effmert et al. 2012). Conversely, growth stimulation capacity toward other species of bacteria, fungi, or oomycetes has also been observed (Schulz-Bohm et al. 2017a; Audrain et al. 2015b).

Most experimental setups have so far characterized the effects of the basal (or constitutive) volatilome emitted by bacteria grown in pure culture (Schmidt et al. 2015; Schulz-Bohm et al. 2017a; Effmert et al. 2012; Avalos et al. 2018). However, how bacteria adapt their volatile production in response to environmental changes is largely unknown, as are the consequences of these modifications on biological interactions. Consequently, effect of interactions on volatile production is currently receiving a special and growing attention from the scientific community.

The production of a volatilome by a bacteria or community of bacteria has been shown to depend on the culture medium (Schulz-Bohm et al. 2017a; Raza et al. 2015; Lazazzara et al. 2017), but as mentioned above, the presence of other microorganisms in the environment will likely affect the volatile emission of each member within the community. The composition of a microbial community itself is largely depending on balance and compatibility between the different strains living in the same media (Hol et al. 2015; Tyc et al. 2015a). So, the composition of this community will also have an effect on the volatiles produced and therefore on the properties of the whole community's volatilome (de Boer et al. 2019).

In this chapter, we address the emergent topic of how interactions between microbes occurring in natural communities shape the emission of volatiles by the community members and which are the consequences of such emission changes on the biological interactions between bacteria and other organisms.

9.2 Influence of Bacteria/Bacteria Interactions on Emission of Volatiles

The volatilome emitted by a community of bacteria is different from the sum of the volatilomes emitted by each individual species within this community (Kai et al. 2018; Schulz-Bohm et al. 2015; Tyc et al. 2015b). The composition is so specific, that monitoring of volatile compounds has been suggested as an efficient method to identify changes in soil microbial communities (McNeal and Herbert 2009). The effect of the interactions between different bacterial species on the volatile blend composition is not precisely known, but recent studies have shown that different associations could lead to opposite effects (Tyc et al. 2017b; Agrawal and Shukla 2016).

Bacteria can strongly influence each other, whether they belong to the same species or not and this association can lead to the production of new volatiles. The effects of many of these interactions have been tested under laboratory conditions and these studies have shown that such interactions could occur starting from the association of two species (Kai et al. 2018; Tyc et al. 2015b, 2017b). The details of the changes in volatilome composition upon interaction between at least two different microbial species are summarized in Table 9.1.

Interactions can lead to the production of specific VOCs: Schulz-Bohm et al. have shown that the co-cultivation of *Burkholderia*, *Dyella*, *Janthinobacterium*, *Pseudomonas,* and *Paenibacillus* grown in media containing four to five of these species led to the production of different volatiles from the ones found in monocultures (Schulz-Bohm et al. 2015). Among the volatiles identified in the blend, several compounds were not detected in monoculture and thus resulted in an enrichment of the volatilome diversity. Among them were notably compounds with known antimicrobial effects such as camphor, 2,5-bis(1-methylethy)pyrazine, and acetic acid.

On the contrary, the co-cultivation of different strains of bacteria can also lead to a loss of volatilome diversity. The co-cultivation of *Chryseobacterium* associated with *Tsukamurella* and co-cultivation of *Janthinobacterium* with *Dyella* led to an increase in the amount of volatiles measured, but did not lead to the production of new volatiles. In addition, several compounds detected in monoculture were absent from the blend emitted by the pairs for both associations (Tyc et al. 2015b). Some highly bioactive volatiles, such as indole (Slininger et al. 2004) and methyl thiocyanate (Ossowicki et al. 2017), were then lost. The same was observed for dimethyl sulfoxide (DMSO), methyl isobutyrate, methyl isovalerate, dimethyl tetrasulfide, and cyclopentene. Since interactions cause significant changes in the composition of the volatilome, they are also likely to alter the effect of a volatilome on microbial communities.

Interestingly, the volatilome emitted by the microbiota from suppressive soils was shown to lose its antioomycete capacity against the oomycete *Pythium intermedium* after partial disinfection; however, this capacity could be recovered over time (van Agtmaal et al. 2015). This suggests that the consequences of a disturbance on the balance of a microbial community are not definitive. Community dynamics seems to

Table 9.1 Effect of interspecific interaction on volatile production

Association	Emitter(s)	Compound	Function	Effect on production	References
Bacteria/ bacteria	Coculture of *Burkholderia*, *Dyella*, *Janthinobacterium*, *Pseudomonas*, *Paenibacillus*	Unknown	n.a.	Emission ON	Schulz-Bohm et al. (2015)
		Methyl 2-ethylhexanoate	Ester	Emission ON	
		2,5-Bis(1-methylethy)-pyrazine	Aromate	Emission ON	
		Unknown	n.a.	Emission ON	
		Unknown	n.a.	Emission ON	
		Unknown	n.a.	Emission ON	
		Unknown	n.a.	Emission ON	
	Coculture of *Burkholderia*, *Dyella*, *Janthinobacterium*, *Pseudomonas*	Camphor	Terpenoid	Emission ON	
		Unknown	n.a.	Emission ON	
		DBQ	Aromate/ phenol	Emission ON	
		Acetic acid	Organic acid	Emission ON	
		Ethyl benzene	Aromate	Emission ON	
		n-hexadecanoic acid	Organic acid	Emission ON	

	Coculture of *Chryseobacterium*, *Tsukamurella*	Methyl thiocyanate		Emission OFF	Tyc et al. (2015b)
		Dimethyl sulfoxide	Antimicrobial	Emission OFF	
		Dimethyl tetrasulfide		Emission OFF	
		Indole	Antimicrobial	Emission OFF	
	Coculture of *Dyella*, *Janthinobacterium*	Cyclopentene	Antifungal	Emission OFF	
		Methyl isobutyrate		Emission OFF	
		Methyl isovalerate		Emission OFF	
	Coculture of *Paenibacillus*, *Burkholderia*	2,5-Bis(1-methylethyl)-pyrazine	Antimicrobial	Up-regulation	Tyc et al. (2017b)
		S-methyl methanethiosulfonate	Antifungal	Emission OFF	
		Pederin like compound	Antimicrobial	Emission OFF	
	Coculture of *Serratia plymuthica*, *Staphylococcus delphini*	Schleiferon	Bactericidal	Emission ON	Kai et al. (2018)
Bacteria/fungi	Coculture of *Staphylococcus pasteuri*, *Tuber borchii*	Bicyclo[2.1.1]hexane-5-carboxylic acid, 5-methoxy-3-methylene-, methyl ester		Emission ON	Barbieri et al. (2005)
		1-Butanol, 3-methyl acetate		Emission ON	
		3-Methyl-1-butanal		Emission ON	
		3-Methoxy-2-cyclopentenone		Emission ON	

(continued)

Table 9.1 (continued)

Association	Emitter(s)	Compound	Function	Effect on production	References
		Dihydro-beta-ionone		Emission ON	
		2(1H)-naphthalenone, octahydro-1-methyl		Emission ON	
		1-Cyclohexanone, 3-butyl-3-methyl		Emission ON	
		5-Methyl-2-hexanol		Emission ON	
		3,3-Dimethyl-2-pentanol		Emission ON	
		1-Buten-3-ol		Emission ON	
		Gamma-patchoulene		Emission ON	
		Eucarvone		Emission ON	
	Coculture of *Serratia plymuthica PRI-2C*, *Fusarium culmorum*	Sodorifen		Emission ON	Schmidt et al. (2017)
	Verticillium longisporum exposed to *Paenibacillus polymyxa* VOCs	1,3-Dioxolane, 2,2,4,5-tetramethyl-, *trans*	n.a.	Up-regulation	Rybakova et al. (2017)
		Pentafluoropropionic acid, hexyl ester	n.a.	Down-regulation	
		Isoamyl acetate	Antimicrobial	Down-regulation	
		Durenol	Possibly antimicrobial	Down-regulation	

		Spathulenol	Plant metabolite	Down-regulation	
		3(2H)-Thiononanoe	n.a.	Up-regulation	
		2-*N*-(2-methylpropyl)Benzothiazolamine		Down-regulation	
	Paenibacillus polymyxa exposed to *Verticillium longisporum* VOCs	3(2H)-Thiononane	n.a.	Up-regulation	
		2-*N*-(2-methylpropyl) Benzothiazolamine	n.a.	Down-regulation	
		2-(4-Cyclohexyl-butanoylamino)-3-chloro-1,4-naphthoquinone	Putative antifungal	Up-regulation	
		Bicyclo (2.2.1)-hepta-2,5-dien-7-ol; or 7-Hydroxynorbornadiene	n.a.	Up-regulation	
		1-Butanol	Antimicrobial	Up-regulation	
		1,2,4-Benzenetricarboxylic acid, 1,2 dimethyl ester	n.a.	Up-regulation	
		Nα, Nω-Dicarbobenzoxy-L-arginine	n.a.	Up-regulation	
		3-Phenyl-5-(benzylthio)isoxazole	n.a.	Up-regulation	
		5,6-Decadien-3-yne,5,7-diethyl	n.a.	Up-regulation	

be a crucial factor for the biological activity of a volatilome. Nevertheless, the loss of some species, even those present in very low abundance, from a community can drastically reduce the suppressive capacity of the community's volatilome.

The most important antimicrobial compounds may apparently be emitted by non-dominant species, i.e., rare, low-abundance microbial strains. These rare bacteria were shown to play a major role in the production of antimicrobial compounds (Hol et al. 2015). The capacity of a community of bacteria to produce an antifungal volatilome was modified after a succession of dilution-to-extinction steps, which led to a rapid reduction in antifungal capacity (Hol et al. 2015). This suggests that despite their number, rare species may play an important role in the production of volatiles, either through direct production or through interactions with more abundant species that could lead to the production of antifungal compounds.

New volatiles can also be produced during competition between two bacterial species (Tyc et al. 2017b). Interspecific interactions can lead to the production of antimicrobial volatiles providing an advantage to the volatile emitter for the colonization of the medium. This kind of interaction has been described between Gram-positive and Gram-negative bacteria. When grown together, both *Burkholderia* and *Paenibacillus* have shown important modifications of their transcriptome (Tyc et al. 2017b). Some of these changes were typical of competitive behavior, e.g., expression of genes involved in the type IV secretion system, in antibiotic resistance, and in cell motility, respectively, leading to secretion of antimicrobial compounds, resistance to antimicrobial compounds, and escape strategy. This interaction also led to the production of 2,5-bis(1-methylethyl)-pyrazine as well as a second unidentified compound, but apparently close to pederin, a highly cytotoxic, antibacterial and antifungal compound (Zimmermann et al. 2009), whose supposed activity lies in cell wall disturbance (Janssens et al. 2019). This compound inhibited the growth of *Burkholderia*, suggesting that *Paenibacillus* was the emitter.

Another study has shown that the volatiles emitted by *Chryseobacterium* and *Tsukamurella* could inhibit the growth of *Escherichia coli* (Tyc et al. 2015b). The authors have also demonstrated that compounds emitted by a monoculture of *Chryseobacterium* and by the co-cultivation of *Dyella* and *Janthinobacterium* could also have a significant effect on the shape of colonies of *Serratia marcescens* even if in this case, the effect on cell viability was not specified.

While multiple studies observed the emergence of new volatiles during bacteria/bacteria interactions, one recent report provided evidence of how such newly emerging compounds could arise, namely by chemical reaction of two precursors emitted by different bacteria into the same atmosphere (Kai et al. 2018). In this work, the production of schleiferon was observed after association between two bacteria of different species. This molecule is an antibacterial compound capable of inhibiting the growth of Gram-positive bacteria and disrupting quorum sensing in Gram-negative bacteria (Lemfack et al. 2016). Schleiferon is normally synthesized in the atmosphere by a spontaneous and non-enzymatic reaction between the two precursors acetoin and 2-phenylethylamine, emitted by *Staphylococcus schleiferi*. However, Kai et al. showed that these precursors were also emitted by *Serratia plymuthica* and *Staphylococcus delphini* (Kai et al. 2018). This study not only

showed that bacteria were able to induce the production of volatiles in other bacteria through signaling, but it also showed that the combined volatilomes themselves could spontaneously generate new compounds with activity against a common threat. However, the authors tempered their results stating that very specific culture conditions are required to trigger the production of these precursors in both species at the same time. These conditions could be too restrictive for this phenomenon to be observed in nature, but could still occur in specific ecological niches (Kai et al. 2018).

The effect of the volatiles emitted by the bacteria is not limited to the inhibition of the growth of other microbial species. It can also trigger growth promotion in other species (Ramirez et al. 2010; Kleinheinz et al. 1999; Fincheira and Quiroz 2018). Some volatiles are known to act as carbon source or growth signaling molecules that can be used by several bacterial species. For example, volatile compounds emitted by pure cultures of *Dyella* stimulated the growth of *Staphylococcus aureus* (Tyc et al. 2015b). But these volatiles could not be metabolized by all bacteria and the same blend could be used for competition or cooperation depending on the target bacteria. For example, exposure to the same blend of VOCs stimulated the growth of *Dyella* and *Burkholderia* while it reduced the growth of *Paenibacillus* when these bacteria were grown under starving conditions (Schulz-Bohm et al. 2015). However, the growth stimulation was not observed when bacteria were grown on a rich medium. On the other hand, the presence of *Paenibacillus* in a bacterial mixture participated to the production of a blend stimulating the growth of *Pseudomonas* and *Dyella*, while inhibiting the growth of *Janthinobacterium* probably through the production of 2,5-bis(1-methylethy)-pyrazine.

Several species of plant growth-promoting rhizobacteria are susceptible to this growth promotion mechanism. When exposed to VOCs produced by the soil, several rhizobia showed better growth (Agrawal and Shukla 2016). Among the three tested soils, only one triggered growth inhibition, and only on a limited number of strains, while all diffusible compounds from this soil stimulated the growth of all bacteria. This suggest that in some cases the effect of volatiles from bacteria could be neutralized by other secreted molecules.

Sulfur-containing volatile organic compounds (sVOCs) can also positively influence the establishment of nodulating bacteria brought to the plant by a nematode vector (Horiuchi et al. 2005). Some sVOCs are known to be potent chemoattractant for nematodes. It has been shown that volatiles produced by the roots of *Medicago truncatula* were capable of inducing chemotaxis in *Caenorhabditis elegans*, a nematode serving as a vector for the symbiont *Sinorhizobium meliloti* (Horiuchi et al. 2005). After analysis and identification of the volatilome, purified compounds were tested and dimethyl sulfide (DMS) was identified as the most potent chemoattractant for the nematode. Once the substance was perceived, the nematode was attracted toward the plant and inoculated the bacteria which improved the level of nodulation of the plant. However, DMS is not only produced by plants, but also by several bacterial species, among which also *Rhizobium* species (Effmert et al. 2012; Ossowicki et al. 2017).

9.3 Influence of Bacteria/Fungi/Oomycete Interactions on Emission of Volatiles

Some volatiles emitted by bacteria are known to effectively inhibit the growth of fungi and oomycetes and their use has been suggested as alternative to synthetic fungicides to fight crop diseases (Raza et al. 2015; Weisskopf 2013; Hunziker et al. 2015; Voisard et al. 1989; Bailly and Weisskopf 2017). Among the most effective volatiles are sVOCs. The volatilome of different strains of *Pseudomonas* was shown to contain three potent members of these sVOCs family that displayed antifungal and antioomycete properties: dimethyl disulfide (DMDS) (Groenhagen et al. 2013), dimethyl trisulfide (DMTS) (Tang et al. 2019), and *S*-methyl methanethiosulfonate (MMTS) (De Vrieze et al. 2015). These sulfur-containing volatiles were able to effectively inhibit the development of *Phytophthora infestans* at all its life stages by acting on both mycelium and spores (De Vrieze et al. 2015). Other volatile compounds emitted by bacteria such as nonanal (Cornelison et al. 2016) have also shown their potency against a wide variety of fungi. This compound is produced by bacteria and has been isolated from fungistatic soils. It has shown a strong growth inhibition capacity against the pathogenic fungus *Ophidiomyces ophiodiicola*. It has been tested in pairs or trios with other pure volatiles identified from the same soil. All combinations including nonanal led to the largest growth inhibition rates that could reach up to 94%.

However, volatiles emitted by fungi and oomycetes can also have an effect on bacteria and affect their volatilome during interactions (Rybakova et al. 2017; Barbieri et al. 2005). Bacteria are not only able to detect the volatiles of other bacteria. They can perceive and react to compounds emitted by organisms from different kingdoms (Del Giudice et al. 2008). Fungi and oomycetes are also producing a wide range of volatiles (Moisan et al. 2019; Morath et al. 2012) and their volatilome is mostly composed of molecules of the same families as those emitted by bacteria, such as terpenes among others. Volatiles emitted by fungi also participate in interspecific exchanges and are, e.g., able to influence the motility of bacteria (Schmidt et al. 2016). As in bacteria, the effect depends on the compound emitted and the target sensibility, resulting in a stimulation or an inhibition. Fungi volatiles can have chemoattractant or repellent effects and may even restrain bacteria motility (Schmidt et al. 2016). For example, the swimming motility of *S. plymuthica* was significantly stimulated or reduced when exposed to the volatilome of the fungi *Fusarium culmorum* and *Rhizoctonia solani,* respectively. However, the effect was different depending on the medium on which the pathogens were grown, probably because different media lead to different volatiles emitted by fungi and oomycetes, similar to what has been observed in bacteria (Insam and Seewald 2010). Swarming motility was also modified for *Collimonas pratensis* after exposure to volatiles emitted by the two fungal species *Mucor hiemalis* and *Rhizoctonia solani*, while its swimming motility was highly inhibited by volatiles emitted by *Trichoderma harzianum* and *Pythium ultimum*. As mentioned above for *S. plymuthica*, these effects varied depending on the medium. Among the blend of volatiles compounds

emitted, it seemed that the effects on motility were mainly due to terpenes such as α-terpinene, β-phellandrene, which showed an inhibitory and stimulating effect, respectively, while 3-carene triggered contrasting effects on *C. pratensis* depending on the concentration (Schmidt et al. 2016): this compound reduced the swarming motility of the bacteria at high concentration but had a stimulating effect when applied in low concentrations. Interestingly, the other phenotypes tested in this study, such as antimicrobial activity, growth promotion, and biofilm formation, were not altered by volatiles emitted by the oomycetes or the fungi, suggesting that the diversity of responses in an inter-kingdom interaction could be more limited than between bacteria.

As observed in bacterial co-cultivation, the volatilome emitted by bacteria in monoculture may be different from that emitted by the bacteria during co-cultivation with a fungus or an oomycete since both partners are able to influence each other (Rybakova et al. 2017). Indeed, after exposure to the VOCs emitted by *Verticillium longisporum*, the production of volatiles by *P. polymyxa* was significantly modified and this interaction led to different kinds of changes: the production of volatiles with indeterminate function was stimulated and in the same time, the production of antimicrobial volatiles such as durenol and isoamyl acetate was inhibited (Rybakova et al. 2017). Similarly, the volatilome emitted by the fungus was modified by the presence of *P. polymyxa* VOCs, which increased the production of several antimicrobial and antifungal compounds such as 1-butanol and 2-(4-cyclohexyl-butanoylamino)-3-chloro-1,4-naphthoquinone. However, even if antimicrobial VOCs production was reduced for *P. polymyxa* after co-cultivation with the fungus, the interaction resulted in a growth inhibition of *Verticillium longisporum* (Rybakova et al. 2017). This suggests that VOCs from *V. longisporum* act as inhibitor toward *P. polymyxa*, but despite this effect the antifungal capacity of the bacteria volatilome remained potent enough to disturb the fungal growth.

An interaction between fungi and bacteria can also lead to the production of new volatiles missing from the blend emitted in monoculture. The volatiles emitted by *Staphylococcus pasteuri* were shown to have a strong antifungal capacity against the mycorrhizal fungus *Tuber borchii* leading to a complete growth inhibition (Barbieri et al. 2005). The analysis of the volatiles emitted by the two partners cultured alone or together showed that among the 65 compounds identified, only 10 were emitted in both monoculture and co-cultivation. Most of them were not detected during the interaction, whereas a dozen was emitted only during the interaction. However, the experimental procedure used in this experiment did not allow to determine which of the two organisms was the emitter (Barbieri et al. 2005). Derivatives of aliphatic, aromatic compounds, terpenoids including γ-patchoulene, the antimicrobial and antifungal compound 3-methoxy-2-cyclopentenone (Ho et al. 2011) (an homolog to the spore germination inhibitor strobilurin A (Sierotzki et al. 2000)), 1-octen 3-ol and 3-methyl butanal were identified among the interaction-specific volatiles, as well as other compounds also emitted by *Mesophellia glauca* and known to be capable of attracting fungus-feeding animals such as *Potorous tridactylus* and *Bettongia gaimardi* (Millington et al. 1998).

Similarly, changes in the volatilome emitted by *S. plymuthica* after exposure to the pathogen *F. culmorum* were analyzed and led to the identification of the terpene sodorifen, produced by the bacteria in response to the interaction with the fungus (Schmidt et al. 2017).

The volatiles emitted by bacteria are not always detrimental. They are also able to stimulate growth and development of fungi, as observed in the case of mycorrhizal fungi. The so-called mycorrhiza helper bacteria (MHB) are able to improve the capacity of roots to recognize mycorrhiza, to enhance the colonization rate, and to stimulate mycorrhizal growth (Garbaye 1994). Although the effect of MHB is not restricted to volatile emission, it was shown that the growth of the ectomycorrhizal fungus *Pisolithus albus* was stimulated by the volatiles emitted by *Pseudomonas monteilii* (Duponnois and Kisa 2006). Mycorrhizal enhancement through bacterial inoculation was observed with *Paenibacillus* and *Burkholderia*, which improved the colonization of *Pinus sylvestris* roots by *Lactarius rufus* (Aspray et al. 2006). However, and surprisingly, the same study also showed that exposure of *P. sylvestris* roots to volatiles emitted by *Paenibacillus* resulted in reduction of mycorrhization rate if the bacteria were not in direct contact with the plant.

Several species of *Streptomyces* belong to the MHB group and are known to improve the germination rate of *Glomus mosseae* spores (Tylka and Axenic 1991), and in the case of *Streptomyces orientalis*, this effect was shown to be mediated by the emission of volatiles (Mugnier and Mosse 1987). However, volatiles emitted by mycorrhizae spore-associated bacteria (SAB) isolated from *Glomus clarum*, displayed the opposite effect. The volatiles emitted by some SAB such as *Bacillus laterosporus* inhibited mycorrhizal spore germination; however, germination could be restored if the same bacteria were in contact with the spore (Xavier and Germida 2003). Among the 35 volatilomes tested from SABs, 13 emitted germination-inhibiting volatiles and nine emitted germination-enhancing compounds, suggesting that different signals of potentially contrasting effects generated by spore-associated bacteria might be involved in triggering or inhibiting spore germination in the soil environment.

9.4 Influence of Volatiles on Bacteria/Protist Interactions

Predatory interactions are key processes in microbial food webs (Geisen et al. 2015). It is well accepted that protists and viruses have major impact on bacterial mortality and herewith on bacterial community composition (Saleem et al. 2013). Volatiles can also play important role in long-distance protists–bacterial interactions (Schulz-Bohm et al. 2017b). By testing various volatile-mediated interactions between phylogenetically different soil bacteria and protists and comparing those with direct trophic interactions, Schulz-Bohm and co-workers demonstrated that bacterial volatiles can provide early information about suitable preys (Schulz-Bohm et al. 2017b). The responses of three different soil protists (*Vermamoeba*, *Saccamoeba,* and *Tetramitus*) to volatiles emitted by six phylogenetically distinct soil bacteria

were tested in a two-compartment Petri-dish system. Bacterial volatiles significantly altered protist activity, motility, and growth. However, the response of protists to bacterial volatiles was strongly dependent on the interacting partner (Schulz-Bohm et al. 2017b). The species-specific responses of protists to bacterial volatiles suggest potential co-evolutionary dynamics in predator–prey interactions. For example, the activity of *Vermamoeba* and *Saccamoeba* was reduced by *Dyella*, whereas *Collimonas* stimulated the activity of *Vermamoeba* and *Tetramitus*. Recently, Song et al. revealed that a *Collimonas* strain emitted volatile terpenes such as β-linalool, β-pinene, germacrene D-4-ol, and δ-cadinene (Song et al. 2015). Interestingly, a *Collimonas* mutant deficient in terpene production failed to alter protist behavior, suggesting that terpenes play a key role in volatile-mediated bacterial–protists interactions (Schulz-Bohm et al. 2017b).

Terpenes constitute the largest class of natural products made by living organisms (Christianson 2017). Most terpenes are known from plants (Chen et al. 2011), but bacteria (Song et al. 2015; Yamada et al. 2015; Dickschat 2016) and fungi (Keller et al. 2005; Schmidt-Dannert 2014) are also rich sources of terpenes. A recent study revealed that dictyostelid social amoebae, a class of eukaryotic soil microorganisms, also have the genetic capacity to produce monoterpenes, sesquiterpenes, and diterpenes (Chen et al. 2016). The amoebal terpene synthases genes show closer relatedness to fungal than to bacterial terpene synthase genes (Chen et al. 2016). Dictyostelid social amoebae have a unique life cycle, consisting of both unicellular and multicellular phases. When their bacterial food supply becomes scarce, amoebae start to aggregate, going through clearly defined morphological changes to eventually form fruiting bodies (Kessin and Dictyostelium 2001). *Dictyostelium discoideum* and *D. purpureum* are among the most extensively investigated species of dictyostelid social amoebae and the multicellular stages of the life cycle of both amoebae are characterized by the emission of a complex mixture of volatile organic compounds dominated by sesquiterpenes. While some terpenoids are produced by both species, most appear to be species-specific (Chen et al. 2018).

The *Dictyostelium discoideum* genome contains nine terpene synthase genes and all nine genes encode active enzymes. Interestingly, transcriptomic and metabolomics analyses revealed that these genes are expressed and terpenes are released in a development-specific manner (Chen et al. 2018; Rabe et al. 2015). The possible biological function of volatile terpenes emitted from *D. discoideum* is yet unknown. However, it is plausible that these terpenes act as signals to coordinate multicellular development and/or to attract their prey. Since reaching the prey can be very costly, protists might have evolved sensory mechanisms to detect bacterial volatiles as early information on the presence of a suitable prey, while the volatiles emitted by the protists themselves could help to attract the prey. Hence, volatile compounds could be of key importance in bacterial–protist predator–prey interactions, although the identity of the signal compounds and their effects on the interacting partners remains largely unknown, as is the influence of bacterial/protist interaction on the emission of volatiles by each partner.

9.5 Concluding Remarks

As highlighted in this chapter, many factors contribute to shaping the dynamic emission of volatiles by bacteria, from abiotic parameters such as nutrient availability to biotic parameters such as the volatile emitter's own changing physiology (e.g., different growth phases depending on nutrient availability) or the presence of different interacting partners in complex communities of organisms sharing the same ecosystem. Beyond purely chemical interactions between molecules emitted by different members of these complex communities, which can give rise to new volatile compounds, most changes in the volatilomes emitted by communities will likely originate from biological interactions: specific compounds emitted by some members will trigger changes in the physiology of other community members, which in turn might lead to altered volatile emission by these affected members (Fig. 9.2). How individual members contribute to the overall constantly changing "smell" of the community, and with which consequences for their own fitness and that of their neighbors is an exciting yet very challenging field of research, in view of the complexity of the underlying biological and chemical interactions.

One particular challenge in deciphering this volatile-mediated multi-species network of interactions lies in the difficulty to link the molecules to their emitters. In contrast to genes, transcripts, or proteins, metabolites have no "signature" of their producer: dimethyl disulfide produced by a bacterium does not differ from that produced by plants or fungi. The use of isotopically labeled compounds, which has proven useful in many more reductionist experimental setups, also does not allow to univocally trace back the metabolites to their producers when considering that bacteria use plant-derived carbon to build their biomass and plants might use bacteria-derived sulfur to build theirs. In addition to this biological challenge of shared metabolites and cross-feeding of precursor molecules, another challenge of technical nature lies in the difficulty to accurately document dynamic changes in complex volatilomes. Most studies available so far have classically sampled volatiles over a certain period of time but systems allowing online monitoring of volatilome shifts, e.g., through online PTR-MS measures, might prove very useful in documenting volatilome changes occurring upon manipulation of abiotic or biotic factors within a complex community.

Moreover, combining such techniques with other "omics" approaches, such as transcriptomics or proteomics might provide an indirect way to assign metabolites to their producing organisms, especially if we manage, in parallel and more reductionist studies on model organisms, to widen our still lacunar understanding of the genetic basis of volatile synthesis. Ultimately, this understanding of the regulation of volatile emission in model organisms and its later integration into more complex systems shall enable us to modulate the communities' overall blend, either by stimulating or inhibiting some members within the community or by providing specific precursor molecules of known pathways leading to the emission of desired volatiles or the repression of undesired volatiles.

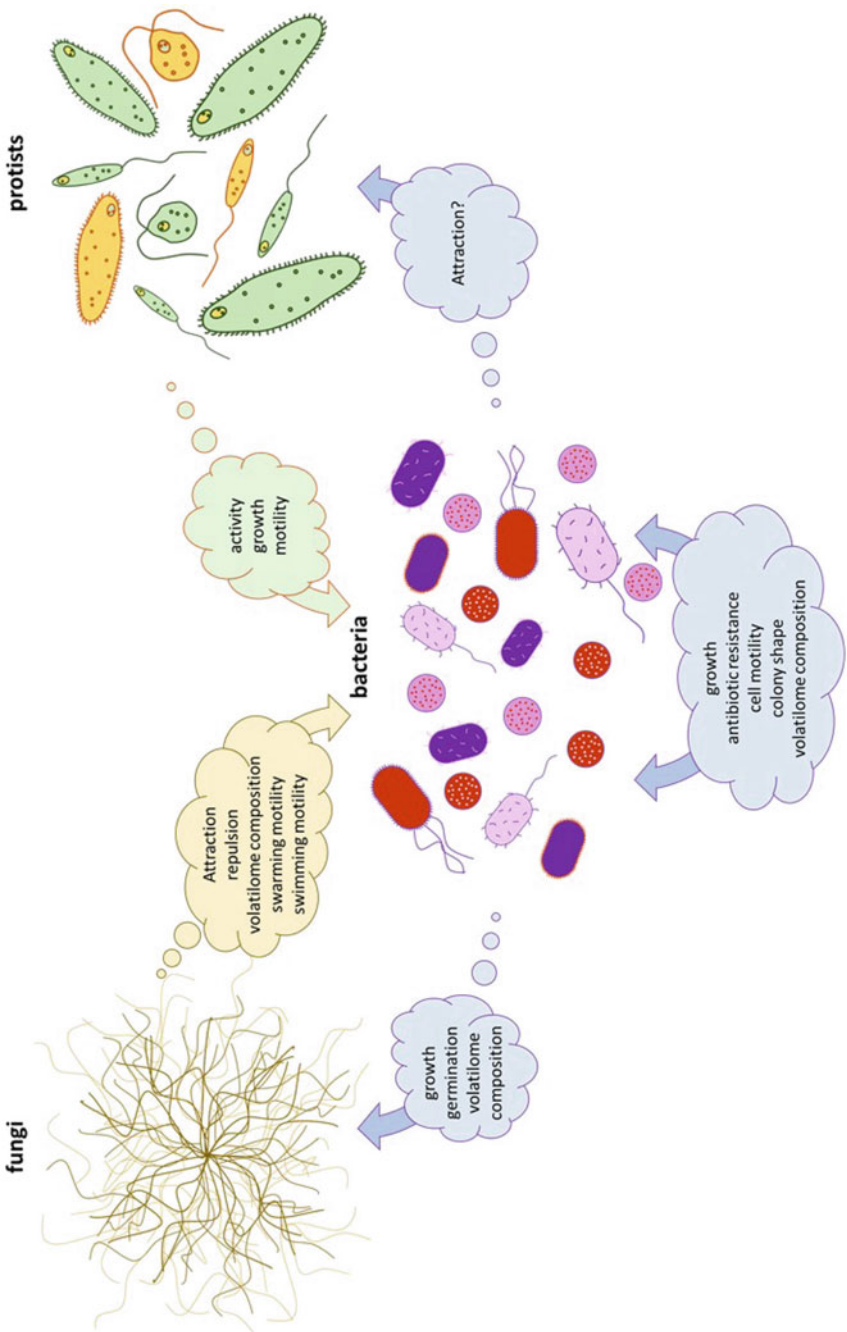

Fig. 9.2 Volatile-mediated interactions between bacteria, fungi, oomycetes, and protists

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Chapter 10
Bacterial Volatiles as Players in Tripartite Interactions

Marco Kai and Uta Effmert

Abstract Microorganisms are powerful but often humble players in the network of intra- and interspecific interactions in ecosystems. Especially bacteria play a crucial role, since they literally can be found in every habitat on earth. This success in colonization and survival rests on their ability to adapt to almost every environment and above that to constantly acclimate to changing environmental conditions. This competence of survival requires a huge variety of strategies and the production of bacterial metabolites involved in signaling, antibiosis, or competition represents one of these strategies to ensure viability. Most interesting but so far rather neglected are volatile bacterial metabolites and their role in intra- and interspecific interactions. The exploration of volatile-mediated interactions involving bacterial species, fungi, arthropods, invertebrates, and the plant reveals astonishing phenomena and relationships. The following chapter will emphasize the distinctive role of bacterial volatiles in tripartite interactions and will give a glimpse of the wealth of fascinating volatile-driven relationships.

Keywords Bacteria · Volatile metabolites · Tripartite · Tritrophic · Interactions · Phytobiome · Insects

10.1 Introduction

Ecosystems represent fragile and complex communities of a broad spectrum of organisms comprising all life forms and habitats. Their natural dynamic stability is driven by a well-balanced system of mutual dependencies realized by intra- and interspecific interactions. These interactions can be specific or non-specific, direct or indirect, but none of them can be considered in isolation. They are embedded in a web of relationships between two, three, or even more partners leading to symbioses,

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co-operation, mutualism, commensalism, competition, antibiosis, or co-existence. Most interesting are effects of an interaction between two parties towards third parties. These so-called tripartite or even multipartite interactions represent a complex system that again generates and affects interactions between trophic levels (Davis et al. 2013; Joshi 2018). This multipartite network is much less understood than the direct interaction between two partners (Stanton 2003). Powerful but often hidden partners in this interplay are represented by microorganisms (Dicke 2008; Dheilly et al. 2015; Shi and Bode 2018; Liu et al. 2019). Bacteria in particular may play an important role, since they are present in probably all ecosystems (Pike et al. 2018). Their success in colonizing a habitat and ensuring their survival in an arising ecosystem depends on their ability to acclimate and adapt to abiotic and biotic factors shaping the environment in short- and long-termed events, respectively. An important role in this struggle for survival can be assigned to bacterial metabolites which are employed as signals in interspecific "cross-talks" or even used as toxins in antibiosis and competition. These molecules should be highly diffusible in order to quickly and reliably reach interaction partners above- and/or below-ground or even in aquatic environments. Volatile metabolites fulfil all of these requirements. Their physicochemical properties (high vapor pressure, low molecular weight, and low boiling point) ensure distribution in all habitats mentioned above (Wheatley 2002; Insam and Seewald 2010; Effmert et al. 2012).

The following chapter will focus on the so far rather neglected role of bacterial volatile metabolites in tripartite interactions. The literature holds numerous reports on volatile-mediated interactions between bacteria and other organisms, such as fungi, plants, and insects, but lacking experimental proof of a tripartite character. Of course, it can be assumed that these interactions will have an impact on other organisms in the corresponding habitat; however, this review will be confined to interactions truly described as tripartite (Fig. 10.1).

10.2 Tripartite Interactions Mediated by Bacterial Volatiles

10.2.1 Interactions in the Phytobiome

The ecosystem consisting of a plant and its surroundings is defined as the phytobiome (Dicke 2016; Leach et al. 2017). Micro- and macro-organisms in this habitat comprise a very high number of diverse taxa including an overwhelming abundance of bacterial species. Bacteria are associated with all plant parts, including roots (rhizosphere), aerial surfaces (phyllosphere), and tissues (endosphere) (Junker and Tholl 2013; Turner et al. 2013; Leach et al. 2017; Arunkumar et al. 2019). Although bacteria populate all plant compartments, the root surface (rhizoplane) shows the highest bacterial diversity and density (Hacquard et al. 2015; Leach et al. 2017). Not surprisingly, the role of the rhizobacterial community in multiple interactions within the phytobiome has been one of the most investigated topics (Pineda et al. 2010). Particularly, the role of plant growth promoting rhizobacteria (PGPR)

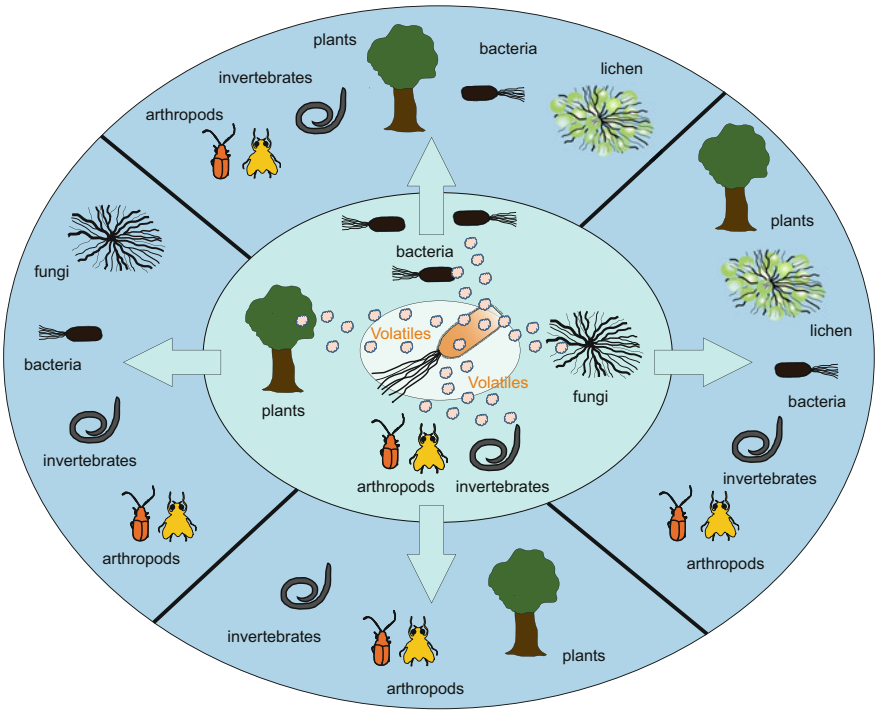

Fig. 10.1 Truly connected: volatile bacterial metabolites as a mediator in tripartite interactions within multi-organismal communities. Bacterial volatiles influence bacteria, arthropods, other invertebrates, and plants (light blue panels). These direct interactions are embedded in tripartite relations and affect other organisms as indicated in the third panel (blue). The figure illustrates volatile-mediated interactions described in the literature and summarized in this chapter

attracts special attention since PGPR represents one of the key players in the complex network of intra- and interspecific interactions within the rhizosphere (Besset-Manzoni et al. 2018).

10.2.1.1 Interplay Between the Plant and Bacteria

Numerous bacterial species of three major phyla dominate the phytobiome (Hassani et al. 2018). Preferences for the same niche, exchange of nutrients, formation of biofilms as well as resource competition and secretion of antibiotic metabolites determine bacterial behavior. They require or trigger cooperative or competitive interactions between bacterial species. The plant might experience beneficial or harmful consequences regarding growth and fitness (Hassani et al. 2018). A bacterial strain that produces antibacterial volatiles is *Bacillus amyloliquefaciens* T-5. Its volatile metabolites reduced the growth of the bacterial pathogen *Ralstonia solanacearum*, which causes wilt in a wide range of host plants. In addition, they

inhibited motility traits, root colonization, biofilm formation, and exopolysaccharide production (Raza et al. 2016). Also the *B. amyloliquefaciens* isolate FZB42 could decrease the size of the colonies, cell viability, and motility of *R. solanacearum* TBBS1 by emitting volatile metabolites including benzaldehyde, 1,2-benzisothiazol-3(2H)-one, and 1,3-butadiene (Tahir et al. 2017a). The volatiles also impaired the chemotactic behavior of the cells and altered the expression level of virulence genes leading to decreased pathogenicity of *R. solanacearum* and less symptoms of the wilt disease in tobacco plants (*Nicotiana tabacum*). Several other *Bacillus* spp. were also effective (Tahir et al. 2017a). Also the volatile metabolites emitted by the *Bacillus subtilis* strain SYST2 were able to cause wilt resistance and growth promotion of tobacco plants (Tahir et al. 2017b). Interestingly, volatile metabolites produced by *R. solanacearum* did not directly affect plant growth, but reduced the effect of plant growth promotion induced by *B. subtilis* SYST2. Studies of co-cultivations of *B. subtilis* SYST2 and *R. solanacearum* revealed that the volatiles of *R. solanacearum* did slightly impair the growth of *B. subtilis* SYST2, but *B. subtilis* SYST2 affected *R. solanacearum* to a greater extent (Tahir et al. 2017b). The reduction in colony size, motility, as well as reduced expression of virulence genes were also observed as a consequence of volatile-mediated interactions between the *Bacillus* strain D13 and the bacterial pathovar *Xanthomonas oryzae* pv. *oryzae*, which causes blight in rice (*Oryza sativa*) (Xie et al. 2018). Rajer et al. (2017) found a similar effect for interactions between *B. subtilis* FA26 and *Clavibacter michiganensis* ssp. *sepedonicus*, which causes ring rot in potatoes. Volatile metabolites emitted by *B. subtilis* FA26 damaged *C. michiganensis* cells and changed colony morphology. Benzaldehyde, nonanal, benzothiazole, and acetophenone revealed antibacterial activity (Rajer et al. 2017).

Beyond their ability to impair growth and pathogenicity of *R. solanacearum*, volatile metabolites produced by *B. amyloliquefaciens* FZB42 and *Bacillus atrophaeus* LSSC22 induced an up-regulation of the expression level of genes involved in wilt resistance and pathogen defense in tobacco plants. These findings indicated the activation of the salicylic acid pathway involved in the induction of systemic resistance in plants (Tahir et al. 2017a). The induction of plant resistance by volatiles is a characteristic trait of certain bacterial isolates. It is a very powerful tool to repress infection with and spreading of bacterial pathogens in plants (Bailly and Weisskopf 2012; Bitas et al. 2013). Pioneering work in this field was done by Ryu and colleagues. They could show as early as 2004 that bacterial volatile metabolites hold a crucial role in inducing systemic plant resistance. Two *B. amyloliquefaciens* isolates (GB03 und IN937) caused ethylene-dependent resistance in *Arabidopsis thaliana* against the necrotrophic pathogen *Pectobacterium carotovorum* subs. *carotovorum* (formally described as *Erwinia carotovora* subs. *carotovora*). Detailed analyses revealed that the volatile 2,3-butanediol was responsible for this effect (Ryu et al. 2004). Other 2,3-butanediol producing bacterial species showed similar effects. *Pseudomonas chlororaphis* initiated resistance in tobacco plants against *E. carotovora* subs. *carotovora* (Han et al. 2006). Most interestingly, Han and colleagues could show that only the 2R,3R-butanediol isomer specifically acted in this way. Furthermore, tobacco plants developed resistance only against *E.*

carotovora, but not against *Pseudomonas syringae* pv. *tabaci*, whereas cucumber (*Cucumis sativus*) plants also built up resistance against *P. syringae* pv. *lachrymans* after exposure to 2,3-butanediol (Song et al. 2019). Song and Ryu (2013) demonstrated in earlier investigations that 3-pentanol und 2-butanone also induced resistance in cucumber against *P. syringae* pv. *lachrymans*. 3-pentanol, produced by *B. amyloliquefaciens* IN937, triggered resistance in pepper (*Capsicum annuum*) against *Xanthomonas axonopodis* pv. *vesicatoria*, which causes bacterial leaf spot on pepper and tomatoes (Choi et al. 2014). Furthermore, 3-pentanol primed plant immunity against *P. syringae* pv. *tomato* via salicylic acid and jasmonic acid-dependent signaling pathways in *Arabidopsis* seedlings (Song et al. 2015). Another interesting volatile metabolite inducing plant resistance is 2-hydroxy-3-butanone (acetoin; Rudrappa et al. 2010). Mutants of *B. subtilis* that emitted lower amounts of acetoin allowed more severe symptoms in *A. thaliana* after *P. syringae* pv. *tomato* DC3000 inoculation than the *B. subtilis* FB17 wild type (Rudrappa et al. 2010). Besides these short-chained compounds, the long-chained aliphatic tridecane also induced plant resistance (Lee et al. 2012; Farag et al. 2013). Lee et al. (2012) showed that infection of *A. thaliana* with *P. syringae* pv. *maculicola* and the outbreak of the disease was much more influenced by *Paenibacillus polymyxa* E681 than by *B. amyloliquefaciens* GB03. The main difference between the two volatile profiles was the emission of tridecane by *P. polymyxa*. Tridecane alone increased the expression levels of salicylic acid, jasmonic acid, and ethylene signaling marker genes (*PR1*, *ChiB,* and *VSP2*, respectively) as much as the volatile mixture. Tridecane also elicited systemic resistance against *P. syringae* pv. *maculicola* in *A. thaliana* (Lee et al. 2012).

10.2.1.2 Interplay Between the Plant, Bacteria, and Fungi

Compared to bacteria, the diversity and abundance of fungi in the phytobiome lags a bit behind (Leach et al. 2017). Nevertheless, fungi also successfully colonize all plant compartments and surroundings where they often closely mingle with bacteria (Kowalchuk et al. 2010; Frey-Klett et al. 2011; Bastías et al. 2020). While competing for nutrients as well as contributing to nutrient production and nutrient cycling, they considerably shape and modulate the habitat, thereby creating new niches and microhabitats (Lynch and Whipps 1990; Bais et al. 2006; Vorholt 2012; Deveau et al. 2018; Schlechter et al. 2019). Bacteria evolved mechanisms that allow them to prevail over fungi. They employ lytic enzymes to destroy fungal cell walls, produce antibiotics to retard fungal growth, or secrete molecules (e.g., siderophores) that capture certain nutritional factors (e.g., Fe^{3+}) (Chet et al. 1990; Kloepper et al. 1980; Compant et al. 2005; Ali and Vidhale 2013; Saha et al. 2016). Research done over the past years revealed that volatile metabolites also play a role in bacterial–fungal interactions. They can affect the hyphal growth as well as the formation and/or germination of spores (Kai et al. 2007; Vespermann et al. 2007; Effmert et al. 2012; Weisskopf 2013; de Boer et al. 2019). Therefore, bacterial volatiles can influence plant–fungal interactions and depending on role and function of the affected fungus

in the phytobiome, the result of these interactions could be beneficial or harmful for the plant.

Many plant colonizing fungal species act as causative agents of plant diseases. These phytopathogenic fungi might interfere with the plant defense, secrete toxins to damage plant tissue, or manipulate the plant metabolism in their favor and even finally destroy their host plant (Doehlemann et al. 2017). The restraint of these processes would be of benefit for the plant. Evaluating the literature, it became obvious that the causal nexus between antifungal activity and plant growth promotion is much less investigated than the direct antifungal effect of bacterial volatiles itself. One of the first reports was published in 1989. Voisard could show that hydrogen cyanide production by *Pseudomonas fluorescens* did control the black root rot of tobacco plants (*Solanum tabacum*), which is caused by the fungus *Thielaviopsis basicola* (Voisard et al. 1989). Fumigation of plants of several species (rice, oilseed rape, and strawberry) with bacterial volatiles reduced disease symptoms caused by *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, and *Botrytis cinerea* in comparison to untreated plants (Wan et al. 2008; Boukaew et al. 2013). Bean plants (*Phaseolus vulgaris* L.) could be protected from infestation with the anthracnose generating fungus *Colletotrichum lindenmuthianum* by application of volatiles, among them 2-methylbutanoic and 3-methylbutanoic acid, emitted by two isolates of *B. amyloliquefaciens* (Martins et al. 2019). Sweet potato (*Ipomoea batatas*) tubers were significantly heavier and showed less disease symptoms of black rot disease in comparison to untreated roots after fumigation of tuber roots with volatile metabolites emitted by *P. chlororaphis* subsp. *aureofaciens* (Zhang et al. 2019). Also, the appearance of blue mold, caused by *Penicillium italicum*, and grey mold, caused by *B. cinerea,* could be suppressed by application of bacterial volatiles of *Streptomyces globisporus* JK-1 in Philippine lime trees (*Citrus microcarpa*) and tomato plants, respectively (Li et al. 2010, 2012).

As already discussed for bacterial pathogens, the crucial question left regarding the phenomena described above is whether a direct antifungal activity of bacterial volatiles or a bacterial volatile-induced systemic resistance of the plant is responsible for the positive effect on fungal pathogen resistance and consequently on plant health and fitness (Compant et al. 2005). Sharifi and Ryu (2016) could show that bacterial volatiles disturbed the fungal attachment to the surface of *A. thaliana* leaves; however, the elicitation of mechanisms responsible for systemic resistance played a more important role. This showed the significant role of bacterial volatiles inducing systemic resistance to prevent undesired fungal spread in plants. Moreover, it could also be confirmed that single bacterial volatiles were able to induce systemic resistance. Huang et al. (2012) demonstrated that the fungal pathogen *Cochliobolus heterostrophus* was not able to cause the typical symptoms of southern corn leaf blight in plants exposed to dimethyl disulfide, which is a widespread bacterial volatile metabolite emitted among others by *Bacillus cereus* C1L. In a similar way, D'Allessandro and colleagues showed that 2,3-butanediol emitted by the endophytic bacterium *Enterobacter aerogenes* induced resistance against *Setosphaeria turcica*, which causes the northern leaf blight in maize (*Zea mays*). They furthermore indirectly proved that the effect exclusively depended on systemic

induced resistance. 2,3-butanediol did not show any antifungal effect against *S. turcica* and *E. aerogenes* did not promote plant growth (D'Alessandro et al. 2014).

However, fungi do not have an exclusively deleterious impact on plants. By far more important to the plant are fungal species that belong to the group of plant beneficial fungi. More than 80% of plant species are associated with the so-called mycorrhizal fungi. Fungal species which undergo a symbiotic relationship with plant roots are most important for the individual plant and its ecosystem. The plant provides the fungus with carbohydrates and in exchange benefits from higher capacity of the fungus to absorb water and minerals (Pickles et al. 2020). Mycorrhiza should be similarly susceptible to bacterial volatiles since the mechanisms of impairment are effective for plant pathogenic as well as for beneficial fungal strains (Giorgio et al. 2015). Indeed, volatile metabolites emitted by the antagonistic bacterial isolate *B. subtilis* JA inhibited the hyphal growth and the germination of spores of mycorrhizal fungus *Glomus etunicatum* (Xiao et al. 2008). Investigations on further consequences due to impaired mycorrhization of plant roots have not been conducted yet. Velivelli et al. (2015) did not directly evaluate the effect of bacterial volatiles on mycorrhization, but they discussed this issue after investigating the antifungal (*R. solani* EC-1) activities and yield increasing properties of PGPR strains (*Pseudomonas palleroniana* R43631, *Bacillus* sp. R47065, R47131, *Paenibacillus* sp. B3a R49541, and *Bacillus simplex* M3–4 R49538) in field trials using potatoes (*Solanum tuberosum*). Another phenomenon was investigated by Aspray et al. (2006). Volatile compounds emitted by *Paenibacillus* sp. EJP73, a so-called mycorrhiza helper bacterium (MHB), hindered the mycorrhizal fungus *Lactarius rufus* to colonize the roots of *Pinus sylvestris*. The MHB was not in direct contact with the fungus and non-volatile factors were missing in the experimental setup that allowed an optimal mycorrhization in the absence of bacterial volatiles. In contrast to the antifungal effect described above, other MHBs, among them a *Bacillus asahii* and a *Methylobacterium* sp. strain, were capable of mycorrhiza growth promotion (Garbaye and Duponnois 1992; Horii and Ishii 2006). Overall, the role of interactions which involve the plant, MHBs, and mycorrhiza for plant growth and health has been acknowledged by now (Pérez-de-Luque et al. 2017; Mohamed et al. 2019; Pathak et al. 2019); however, the role of bacterial volatile metabolites in these interactions awaits deeper investigations.

10.2.1.3 Interplay Between the Plant, Bacteria, and Arthropods

Similarly to the direct effect of bacterial volatile metabolites on bacteria and fungi, they also have a direct impact on arthropod behaviors. Bacterial volatiles can mimic pheromones, stimulate oviposition, or serve as food attractants (Davis et al. 2013). These phenomena have consequences for the plant. In addition, the abundance of certain arthropods in the phytobiome can cause significant problems accompanied with severe loss of metabolic capacity and fitness of the plant (Funderburk et al. 1993). Therefore, the implication of bacteria in plant defense mechanisms against pests has been an important research topic, as discussed below. Although most

studies did not examine the role of bacterial volatile metabolites in particular, we still mention these examples in this chapter, because we consider it very likely that volatiles may be involved in at least some of the effects described, and all examples give rise to further profound investigations.

Whereas *Rhizobium leguminosum* improved the performance of chewing (cotton leaf worm, *Spodoptera littoralis*) and had a neutral effect on sucking insects (green peach aphid, *Myzus persicae*) (Kempel et al. 2009), the colonization of the rhizosphere with PGPR mostly showed a negative impact on chewing as well as sucking insect pests. *B. amyloliquefaciens*, *B. subtilis*, and *B. pumilus* reduced the silverleaf whitefly (*Bemisia argentifolii*) infestation and thereby the *Tomato mottle virus* disease severity in tomato (*Solanum lycopersicum*) plants (Murphy et al. 2000). Furthermore, root inoculation of tomato plants with *B. subtilis* reduced the development of adult cotton whiteflies (*Bemisia tabaci*) from pupae. This resistance seemed to be jasmonic acid (JA) independent, since the pupae development was still retarded in a tomato mutant impaired in wound-induced JA synthesis. In contrast, the induced simultaneous expression of genes encoding for protease and proteinase inhibitors also suggested a JA-dependent resistance. Proteinase and protease inhibitors counteract chewing activities of pupae and support an anti-nutritive effect (Valenzuela-Soto et al. 2010). Cucumber seeds treated with *B. pumilus, Flavimonas oryzihabitans*, *Pseudomonas putida*, and *Serratia marcescens* strains produced seedlings which were more resistant to spotted and striped cucumber beetles (*Acalymma vittatum* and *Diabrotica undecimpunctata howardi*, respectively) and showed less symptoms of the bacterial wilt caused by *Erwinia tracheiphila* transmitted by the beetle (Zehnder et al. 1997a, b). By contrast, supplementations of the potting soil with *B. amyloliquefaciens* and *B. subtilis*-containing products or *P. fluorescens* suspension prior planting of sweet pepper seedlings or *A. thaliana*, respectively, were not effective against aphid infestations (Herman et al. 2008; De Vos et al. 2007). However, preparation of rice seeds or spraying with a *P. fluorescens* containing formula consequently reduced the incidence of leaffolder (*Cnaphalocrocis medinalis*) pests (Commare et al. 2002; Saravanakumar et al. 2007). This formulation was also very effective at controlling sucking pests (leafhopper, *Amrasca biguttula biguttula* and melon aphid, *Aphis gossypii*) on okra (*Abelmoschus esculentus*) plants after seed preparation. Inoculation of *P. fluorescens* into potting soil and subsequent colonization of plant roots by the PGPR resulted in an altered emission of plant volatiles and increased attraction of the parasitoid wasp *Microplitis mediator* to plants (*A. thaliana*) infested by larvae of the cabbage moth (*Mamestra brassicae*) (Pangesti et al. 2015). This effect was accompanied by disturbed growth and development of the generalist *Spodoptera exigua* (beet armyworm) feeding on *A. thaliana* plants due to the induction systemic resistance. The specialist *Pieris rapae* (cabbage butterfly) was not harmed (Van Oosten et al. 2008). Furthermore, the larval development and oviposition behavior of *Spodoptera frugiperda* (fall armyworm) were impaired by application of eight different blends of PGPR including *B. amyloliquefaciens*, *B. atrophaeus, B. circulans, B. pumilus, B. sphaericus, B. subtilis, Brevibacillus brevis, B. laterosporus,* and *Paenibacillus macerans* to Bermuda grass roots (Coy et al.

2017). An example of employment of a single bacterial derived volatile metabolite was given by Song and Ryu (2013). 2-Butanone, shown to be produced by plant root-associated *Bacillus* ssp., triggered systemic resistance in cucumber seedlings which caused an effective defense response against *P. syringae* pv. *lachrymans* (see Sect. 10.2.1.1). In addition, the up-regulation of the cucumber lipoxygenase activity and the subsequent green leaf volatile emission of the plant led to the attraction of lady bird beetles (*Coccinella septempunctata*) which significantly reduced the number of sucking green peach aphids (*M. persicae*) on the cucumber seedlings (Song and Ryu 2013).

Despite their overwhelming number and diversity, rhizobacteria and their volatile metabolites are not alone in driving interactions between species. The phyllosphere also harbors a distinct bacterial community which has to endure strong fluctuations in nutrient supply and environmental conditions (Leach et al. 2017). The removal of the floral microbiome caused a decrease in emission of terpenes from *Sambuca nigra* flowers as well as an altered composition and proportion of terpenes in the mixture (Peñuelas et al. 2014). *Brassica napus* flowers associated with epiphytic bacteria showed an altered emission of volatiles compared to sterile flowers. The presence of a mixture of six intrinsic and re-inoculated bacterial strains induced or reduced the emission of certain volatile components (Helletsgruber et al. 2017). This bacterial interference on floral chemical signaling is not without consequences. *B. napus* flowers inoculated with strains of *Pantoea agglomerans* and *P. syringae* were indeed preferred by pollinators. This could be explained by a changing quality of the floral bouquet due to participation of bacterial volatiles, and reduced emission of some floral volatiles (Farré-Armengol and Junker 2019). Furthermore, Farré-Armengol and his group found out that bacterial inoculations changed the volatile emission of bean leaves (*P. vulgaris*). This response affected interactions between the plant and spider mites (*Tetranychus urticae*). The mites avoided leaves sprayed with *P. syringae* pv. *syringae* B728a or *P. putida* EU275365 and preferred leaves colonized with *Pantoea ananatis* 26SR6. Leaves inoculated with *P. syringae* subsequently showed lower feeding damage and a decreased egg load compared to control (non-sprayed) and *P. ananatis* inoculated leaves (Karamanoli et al. 2020). Microorganisms including bacteria were also found in nectar. These nectar-inhabiting microbial communities seem to be common but very variable in composition. They produce distinct blends of volatiles which affect generalist pollinator preferences. Honey bees were able to distinguish between distinct volatiles. The most preferred nectar-inhabiting microorganism though was *Metschnikowia reukaufii*—a yeast fungus producing ethanol (Rering et al. 2017).

Knowledge about the impact of bacterial endophytes on tripartite interactions is rather scarce. The role of *E. aerogenes*, an endophyte isolated from maize (*Z. mays*) was contradictory. On one hand, plants grown in *E. aerogenes* inoculated soil were less resistant against the herbivory of *S. littoralis* larvae. On the other hand, supplementation of potting soil with 2,3-butanediol, a major component of the volatile mixture produced by *E. aerogenes*, caused an increased attraction of the parasitoid wasp *Cotesia marginiventris* which is known to attack *S. littoralis* larvae (D'Alessandro et al. 2014). Application of 2,3-butanediol to the headspace of the

plant had no effect. This again corroborates the central role of the rhizosphere within the network of interspecific interactions in the phytobiome.

The last example turns the causal relationship described so far upside down. Infestation of pepper plants (*C. annuum*) with green peach aphids (*M. persicae*) modulated the composition of root exudates which caused a notable attraction of *Paenibacillus* spp. and subsequently an increasing colonization of the pepper rhizosphere. Root exudates secreted from aphid infested pepper plants increased growth rates of the *P. polymyxa* strain E681, but surprisingly, the application of *P. polymyxa* E681 to pepper roots accelerated the aphid infestation (Kim et al. 2016). This seems to be an example for a self-facilitated arthropod infestation. The arthropod manipulated the plant to modulate its rhizosphere colonization, which in turn modulated the pest resistance of the plant.

10.2.2 Interactions Between Bacteria and Fungi

Bacteria can actively or passively move around to colonize nutrient rich zones or to escape "inconvenient" areas. Driven by flagella or pili, they directly move on surfaces using mechanisms such as swimming, swarming, gliding, and twitching (Henrichsen 1972; Harshey 2003). However, in order to efficiently disperse, they depend on passive "hitch-hiking" on motile organisms such as protists and fungi that are able to cover longer distances (Hassani et al. 2018). It could be shown that bacteria can attach to fungal hyphae (Kjeldgaard et al. 2019) and that they use the mycelial network as a "highway" (Kohlmeier et al. 2005, Simon et al. 2015, Worrich et al. 2016 in Hassani et al. 2018). This literally "paves the way" for bacteria to reach formally spatially separated bacterial interaction partners. In addition, the fungal mycelium facilitates horizontal gene transfer between spatially separated bacteria (Berthold et al. 2016). The role of bacterial volatile signaling compounds in these interactions has not been examined yet. The same applies to the question of how bacteria-derived antifungal activity within the habitat would influence these interactions as, i.e., retardation or even impairment of fungal growth would hinder bacterial dispersal. However, these phenomena should be introduced here, since they represent another interesting research field regarding tripartite microbial interactions.

10.2.3 Interactions Between Bacteria, Fungi, and Lichen

Lichen represents a mutualistic relationship between fungi and an organism capable of photosynthesis, usually cyanobacteria or algae (Richardson 1999). Due to this symbiosis, the lichen withstands most harsh environmental conditions (Armstrong 2017). Interactions with bacteria provide additional substantial benefit, since these interactions protect the community from damaging fungi and bacteria (Cernava et al. 2015a). Recently, Cernava et al. (2015b) could show that bioactive volatile

compounds produced by lichen associated bacteria repelled the fungus *B. cinerea* (Cernava et al. 2015b). The question, to what extent this bacterial antimicrobial effect influences survival and well-being of the lichen should be a starting point of further investigations.

10.2.4 Interactions Between Bacteria, Fungi, and Invertebrates

The colonization of microhabitats by invertebrates such as nematodes, bacteria, and fungi creates numerous opportunities for interactions and the role of bacteria here is not only restricted to serve as prey for bacterivorous nematodes. Wang et al. (2014) could show that bacteria developed a mechanism to eliminate their predators. Urea released by bacteria (*Stenotrophomonas maltophilia* CD52) served as an intermediate signal to mobilize fungi (*Arthropodus oligospora*) nearby to form specialized hyphal structures (traps) to capture nematodes and utilize them as food source. The signal molecule to initiate the switch from a saprophytic to the predacious fungal stage was found to be ammonia, a degradation product of urea. Ammonia emerges upon up-take and metabolization of urea by the fungus or by external metabolization since an *A. oligospora* mutant lacking urease activity was not able to induce trap formation, but supplementation of the mutant and the wild type with external ammonia restored and induced the predacious stage, respectively. The urea non-producer *B. amyloliquefaciens* was not able to induce trap formation. These findings could clearly indicate a tripartite relationship in soil where urea-producing bacteria mobilize nematode-trapping fungi via ammonia to get protection against their predators (Wang et al. 2014). Ammonia was used as signal molecule by several other bacterial species attracting several other fungal species as trap-forming partner like *Arthrobotrys guizhouensis, Dactylellina phymatopaga, Dactylellina cionopaga,* and *Drechslerella brochopaga* (Su et al. 2016).

Fungi using arthropods as food source but acting as parasites are so-called entomopathogenic fungi. The loss of these fungi may lead to an improved growth of the non-infested arthropod, but may invite new invaders that can feast on a healthy prey. It has been shown that nematodes (*Oscheius* sp.) inject their symbiotic bacteria (*Alcaligenes faecalis*) into an arthropod (*Galleria mellonella*). Although in this case the bacteria were observed to kill the arthropod, the release of volatiles inhibited the growth of the entomopathogenic fungi (*Mucor circinelloides*, *M. racemosus*, and *Rhizomucor variabilis*). The nematodes, colonized by the bacterial symbiont, could safely invade *G. mellonella*, did not have to compete longer with the fungus, and had extended benefit from the dead arthropod as food source (Shan et al. 2019). The fungus *Ophiostoma minus* and eight bacterial species including *Roseateles aquatilis*, *Serratia liquefaciens*, and *Pseudomonas* sp. were part of an equally interesting interaction. Both the bacteria and the fungus are symbionts of bark beetle larvae (*Dendroctonus valens*). Under certain nutrient conditions, the fungus turns into a

competitor causing dramatic weight loss of the larvae. Volatile metabolites emitted by the symbiotic bacteria reduced the fungal growth, which in turn supported the larval development (Wang et al. 2017). The same authors found that the symbiotic bacteria might interfere with the carbohydrate preference of the fungal co-symbiont *Leptographium procerum* of *D. valens* larvae. Ammonia emitted by symbiotic bacteria hindered the fungal utilization of the naturally preferred D-glucose and promoted the fungal use of D-pinitol creating thereby optimal nutrient condition for larvae, which now could utilize the D-glucose in the first place (Zhou et al. 2016, 2017). However, the symbiont could also be the enemy inside. As described in Boone et al. (2008), different stages of the North American pine engraver (*Ips pini*) host fungal (*Ophiostoma ips* and *Pichia scolyti*) and bacterial (*Burkholderia* spp.) species. These organisms provide volatile chemical cues that facilitate the attraction of parasitoids (*Heydenia unica*) and dipteran predators (*Rhopalicus pulchripennis*, *Medetera* sp.) feeding on *I. pini*. The pine tree, again, might benefit from these interactions.

10.2.5 Interactions Between Bacteria and Arthropods

Bacterial volatiles might also drive tripartite interactions that could be employed in pest control. Aphids represent one of the most serious pests in agriculture worldwide (Sadeghi et al. 2009). Leroy et al. found bacteria in the honeydew of pea aphids (*Acyrthosiphon pisum*) which are responsible for the attraction of the hoverfly (*Episyrphus balteatus*). This fly is the most efficient aphid predator, since its larvae also feed on aphids. Among the bacterial isolates found in the dew, the species *Staphylococcus sciuri* could be identified as producer of the volatile semiochemicals that lured the hoverfly to feed on aphids and stimulated oviposition. Volatiles exclusively emitted by *S. sciuri* were 2-methyl-2-buten-1-ol, 3-methyl-2-butenal, and 2- and 3-methylbutanoic acid. The plant has been observed to be a silent partner so far, but it benefits as fourth party from the aphid suicide (Leroy et al. 2011). Ants could help to avoid this fatal attraction. Mutualistic interactions between ant and aphid species are well known. Ants feed on the carbohydrate-rich honeydew and repel aphidophagous predators (Stadler and Dixon 2005; Yao 2014). Fischer et al. (2015) could show that black garden ants (*Lasius niger*) were attracted not only to the honeydew produced by black bean aphids (*Aphis fabae*), but also to volatile metabolites of bacteria originally found in the honeydew. Six bacterial strains were isolated. The semiochemical blend produced by *Staphylococcus xylosus* was most effective in attracting ant scouts. Twenty-four volatile compounds were identified from the honeydew and most of them were also detected in *S. xylosus* cultures. Some of them were also produced by *S. sciuri* mentioned above. However, *S. xylosus* did not produce 2-methyl-2-buten-1-ol and 3-methyl-2-butenal (Fischer et al. 2015).

10.3 Conclusions and Perspectives

As this collection of bacterial volatile-mediated biotic interactions already implicates, microorganisms and especially bacteria can be considered as linking elements in most ecosystems. The presence of microorganisms probably turns most interactions in ecosystems into tri- and even multipartite interactions comprising all possibilities from mutualism and symbiosis to parasitism and feeding activities. In large part, these interactions still remain as a secret web of dependencies between competitors and allies. The knowledge gained so far is derived from reductionistic and mechanistic approaches. Then again, ecological studies give us an idea of the holistic character of microorganism-mediated interactions in ecosystems (Dheilly et al. 2015; Finkel et al. 2017; Vandenkoornhuyse et al. 2015). On that score, the nature of microorganism-mediated interactions in ecosystems remains poorly understood. Challenging aspects in future investigation using a holistic or "eco-holobiont" approach will comprise identification of holobiont components, assembly of this information to form the holobiont, and revealing the mechanisms and functions including the chemistry of multipartite reliances and interactions that keep the ecosystem in balance (Liu et al. 2019; Simon et al. 2019). Tools and techniques to acquire the data and information are available, but most challenging will be the task of sorting through the information to unravel causal key links. Another challenging fact will probably be the natural fluctuation and variation in composition and function of host microbiomes. Also, abundance and importance of host-associated microbiomes can vary (Hammer et al. 2019). Complementary reductionistic investigations of specific phenomena will still be indispensable in order to find substantiating evidence for concrete interaction and causal relations. The holistic approach, however, to explore ecosystems creates at its best an understanding of a functioning and healthy ecosystem. This knowledge could be introduced to promote agriculture and nature conservation. A good example in this regard represents the knowledge accumulated about the plant holobiont—the phytobiome (Hassani et al. 2018; Vandenkoornhuyse et al. 2015). The understanding of the chemical dialogue and functional mechanisms that drive the phytobiome represents a key issue to employ, e.g., plant-associated beneficial bacteria to increase sustainability in agriculture and biological pest management (Neveu et al. 2007; Turner et al. 2013; Finkel et al. 2017; Thomashow et al. 2019) or phytoremediation (Singer et al. 2004).

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Chapter 11
Cyanobacterial VOCs as Allelopathic Tools

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Abstract Cyanobacteria are photoautotrophic microorganisms; some of them are able to fix atmospheric nitrogen. They play an important role in diverse ecological systems. Cyanobacteria produce a wide variety of secondary metabolites with miscellaneous biological functions. Some of these molecules are harmful toxins that affect animals and humans health. Chemical ecology focused so far on the properties of cyanotoxins. However, this research offers important insight into the functional roles of volatile organic compounds (VOCs) and the intra- and extracellular controls of their production. Cyanobacteria are one of the main sources of VOCs, which cause disgusting odor and taste in drinking and recreational water. As far as we know, cyanobacterial volatiles have a significant impact on the physiological functions of prokaryotic and eukaryotic organisms. VOCs excreted by cyanobacteria (geosmin, 2-MIB, β-ionone, β-cyclocitral, monoterpene alcohols, aliphatic fatty acids, aliphatic alcohols, aldehydes, and others) act as allelopathic agents towards phytoplankton. Some cyanobacterial VOCs can act as both the powerful repellents and as "poor quality food" signals to grazers. These small secondary metabolites have a pronounced effect on the competition between microorganisms and hence they are ecologically very important.

Keywords Cyanobacterial VOCs · Geosmin · 2-Methyl-isoborneol · β-Cyclocitral · Allelopathic agents · Odorous compounds · Receptor · Competition · Chemical ecology · Phytoplankton

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11.1 Introduction

Cyanobacteria are photoautotrophic ancient prokaryotic microorganisms that are model objects to study different fundamental processes: carbon and nitrogen metabolism; hydrogen production; N_2 fixation and heterocyst formation; resistance to different stresses; and plant evolution. Numerous genetic tools were developed and applied for filamentous and unicellular strains of cyanobacteria (Koksharova and Wolk 2002). They play a fundamental role in diverse ecological systems (Potts and Whitton 2000; Rai et al. 2002). The natural plasticity and adaptability of cyanobacteria are largely determined by their ability to synthesize various metabolites (Wase and Wright 2008). The diversity of these molecules, including those of biotechnological importance, is amazing (Kamravamanesh et al. 2018). Furthermore, cyanobacteria produce volatile organic compounds (VOCs) that affect taste and odor of drinking and recreational water (Watson 2003; Jüttner and Watson 2007; Watson et al. 2016; Lin et al. 2019). The synthesis of numerous secondary metabolites of cyanobacteria, including various VOCs, is an energy-consuming process; therefore, bacteria use these compounds as adaptive tools in response to environmental changes. These VOCs have potential to act as "infochemicals" and/or "allelochemicals." Cyanobacterial volatiles have a significant impact on the physiological functions of prokaryotic and eukaryotic organisms. Some VOCs such as β-cyclocitral, α-ionone, β-ionone, limonene, eucalyptol, and geranylacetone act as allelopathic agents towards the phytoplankton. It has become apparent that these metabolites may be functioning in a variety of chemical interactions in the aquatic ecological niches as attractants, deterrents, protectants, and toxins (Höckelmann et al. 2004; Watson et al. 2007; Jüttner et al. 2010; Zuo 2019). However, the ecophysiology and chemical ecology of cyanobacterial VOCs are still not well understood and therefore they are fascinating areas of research.

11.2 VOCs Produced by Cyanobacteria

The major groups of VOCs produced by cyanobacteria that have been studied so far are terpenoids, carotenoid derivatives, fatty acid derivatives, and sulfur compounds (Watson 2004). Cyanobacteria synthesize geosmin, 2-methyl-isoborneol (MIB) (Gerber 1977; Zhang et al. 2009), pentan-3-one, octan-3-one, beta-cyclogeraniol (Höckelmann and Jüttner 2004), hydroxyketones, hexane-2,3-diol (Höckelmann and Jüttner 2005), dimethyl sulfide (DMS) (Carrión et al. 2015), and other VOCs (Jüttner 1987; Ali 2004; Achyuthan et al. 2017). These VOCs are not produced by all species or strains of cyanobacteria, and they are also not unique to these taxa but are produced by other microorganisms such as actinomycetes and fungi, which inhabit the same niche making it difficult to identify the precise source of VOCs (Bruchet 1999). A microbial volatiles database (mVOC 2.0) (Lemfack et al. 2018) is available now at: http://bioinformatics.charite.de/mvoc.

The two secondary terpenoids (geosmin and 2-MIB) are considered as the most problematic and ubiquitous biogenic odor compounds reported from freshwaters (Jüttner and Watson 2007). The geosmin and MIB release depends on the cyanobacterial growth phase and different environmental factors (Wu and Juttner 1988b; Naes et al. 1985, 1988; Wu et al. 1991; Zimba et al. 1999; Zhang et al. 2009; Watson et al. 2016). These VOCs are produced by diverse planktonic and terrestrial cyanobacteria, many of which are likely as yet unidentified (Watson et al. 2016). The terpenoid structure of geosmin was first characterized by Bentley and Meganathan (1981). Authors proposed that it was likely a degraded sesquiterpene produced via the mevalonate (MV) isoprenoid pathway. The second biosynthetic route, the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway (also known as 1-deoxy-D-xylulose-5-phosphate pathway), was also implicated (Flesch and Rohmer 1988) in geosmin biosynthesis. In the case of cyanobacteria, MEP pathway utilization has been demonstrated in *Synechocystis* (Proteau 1998; Disch et al. 1998a, b; Kuzuyama 2002). It was shown that cyclization of farnesyl diphosphate to geosmin is catalyzed by geosmin synthase via three steps (farnesyl diphosphate to germacradienol, germacradienol to 8,10-dimethyl-1-octalin, and 8,10-dimethyl-1-octalin to geosmin) in cyanobacteria (Giglio et al. 2008).

Until 1975, MIB was known to be a metabolite of only certain actinomycetes, and then other species that were capable of releasing MIB were identified (Smith et al. 2008). Among them are *Oscillatoria variabilis*, *Hyella* sp., and *Jagerinema genimatum*. The MIB synthesis in cyanobacteria includes two biochemical reactions (Giglio et al. 2011). First, geranyl diphosphate 2-C-methyltransferase (GPPMT) catalyzed methylation of geranyl diphosphate, C10 monoterpene precursor, into 2-methylgeranyl diphosphate. Second, MIB synthase (MIBS) catalyzed cyclization of the 2-methylgeranyl diphosphate to MIB. In the absence of genome sequence data from 2-MIB producing cyanobacteria, Giglio et al. (2011) employed complete genome sequencing of a 2-MIB-producing *Pseudanabaena limnetica* (Castaic lake) to show the presence of sequences similar to MIBS and GPPMT.

The geosmin and 2-MIB are released during the death and lysis of cyanobacterial cells. Subsequently it is difficult to identify both compounds in environmental samples. In addition it is sometimes difficult to isolate and maintain pure cyanobacterial strains. Nowadays intensive metagenomic studies have been performed to elucidate the genes and enzymes involved in the metabolic routes of geosmin and MIB production. These bioinformatic data are used to quantify the geosmin/MIB production potential in various aquatic environments and to elucidate the biochemical mechanisms responsible for the VOCs production (Otten et al. 2016). Geosmin/MIB-producing cyanobacteria were also explored by polymerase chain reaction (PCR) methods to unravel target genes encoding geosmin synthase and MIB synthase (Jüttner and Watson 2007; Giglio et al. 2008; Kim et al. 2014; Suurnäkki et al. 2015; Wang et al. 2015). The quantitative polymerase chain reaction (qPCR) allowed to detect the mRNA levels of respective genes in *Anabaena* sp. cells (Su et al. 2013). A linear relationship between the number of cells and MIB concentration based on the MIB synthase gene expression was reported (Wang

et al. 2016). Recently, a bioelectronic "nose" for the real-time assessment of geosmin and MIB was also reported (Son et al. 2015).

β-Cyclocitral (a tobacco-smelling substance of the nor-carotenoid group) is often found in eutrophic freshwaters, which is the most dominant VOC of cyanobacteria *Microcystis* (Jüttner et al. 2010; Ozaki et al. 2008). Also, it is known that *Microcystis aeruginosa* causes a fruity odor resulting from β-ionone (Zhang et al. 2016). Alkyl sulfides and the β-carotene derivative β-cyclocitral often produce these odors in surface waters; certain noxious cyanobacteria species are the major sources of these compounds (Jüttner 1984). β-cyclocitral and its oxidation products together with β-ionone were significantly detected in highly dense cultures of *Dolichospermum* spp. and *Microcystis* (Arii et al. 2018).

VOC profiles of the heterotrophic cyanobacterium, *Phormidium autumnale*, were studied using axenic cultures in a bubble column growth bioreactor (Santos et al. 2016). Altogether 68 VOCs were identified during this study, while 3-methyl-butanol was found as the major volatile. Furthermore, several terpenoid VOCs, including β-ionone, β-cyclocitral, and 5,6-epoxy-β-ionone, along with VOCs from the 2-keto acid pathway such as 3-methyl-butanol, propanol, butanol hexanol, and hexanal were present. The most abundant VOCs were ketones, such as 2,3-butanedione and dihydro-2-methyl-furanone. Aldehydes were fewer in the VOC profile. In the headspace only linear short chain (C3–C5) alcohols were detected. Such alcohols are characteristic biomarkers for cyanobacteria. Among acidic volatiles, acetic acid dominated followed by butanoic acid, isobutyric acid, and isovaleric acid (Santos et al. 2016). This analysis of the volatile profile is a good start to VOC metabolome of in vivo cyanobacteria.

In another study, VOCs from the filamentous, planktonic cyanobacteria *Spirulina platensis*, *Anabaena* spp., and *Nostoc* spp. were analyzed using GC-MS (Milovanović et al. 2015). 2-MIB was present in these three cyanobacteria, while geosmin was not detected in all samples. Among a total of 17 VOCs identified, the main VOCs were medium chain length alkanes, 2-pentyl-furan, β-cyclocitral, and β-ionone. The principal component analysis (PCA) along with hierarchical cluster analysis (HCA) segregated microalgal species based on VOC profiles (Milovanović et al. 2015). Such pattern recognition tools must play a key role in the analysis of microalgal VOCs and microalgal metabolome.

Filamentous cyanobacteria *Calothrix*, *Phormidium* sp., *Plectonema*, and *Rivularia* sp. produced a broad range of polyunsaturated fatty acid (PUFA) derivatives, including 1,3,3-trimethyl-2,7-dioxabicylo(2,2,1) heptane (TDH) and 6-methyl-5-hepten-2-one (Höckelmann and Jüttner 2005). Bromomethane (CH_3Br) and isoprene are produced by *Synechococcus* sp. (NIES-981) (Hiraiwa et al. 2014).

The biogenic sulfur VOCs implicate in the global sulfur cycle (Bentley and Chasteen 2004) and in climate (Watson and Jüttner 2017). Approximately 50% of the biogenic sulfur is emitted into the atmosphere annually by algae (Van Alstyne and Houser 2003). Cyanobacteria emit such alkane thiols as methanethiol and isopropylthiol. *M. aeruginosa* has been identified as a major emitter of isopropylthiol (Watson and Jüttner 2017). This cyanobacterial species is a

bloom-forming toxic microorganism, which causes substantial ecological and economic damage (Oberholster et al. 2009). It is proposed that monitoring of the emission of VOCs might contribute to early detection of cyanobacteria, thus enabling preventive actions to be implemented.

Different sulfur compounds are produced by many cyanobacteria. So, dimethyl sulfide (DMS) can be released by different cyanobacteria, such as *Anacystis nidulans*; *Synechococcus cedrorum*; *Oscillatoria chalybea; Oscillatoria tenuis; P. autumnale; Plectonema boryanum*, whereas dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), isopropyl disulfide are produced by different species of *Microcystis* (Lee et al. 2017). Only cyanobacteria *Calothrix parietina*, *Rivularia* sp., *Tolypothrix distorta* produce ketone and ionone derivatives (Höckelmann and Jüttner 2005; Smith et al. 2008).

The cyanobacterium *M. aeruginosa* NIES-843 produces different alcohols, such as 2-methyl-1-butanol, 3-methyl-1-butanol, and 2-phenylethanol, using the 2-keto-acid decarboxylase (Hasegawa et al. 2012). Several classes of halomethanes were produced by different types of cyanobacteria and other microalgae (Paul and Pohnert 2011).

It is noteworthy that the spectrum of volatile organic compounds formed by cyanobacteria differs from the VOCs formed by eukaryotic algae. The type of VOCs in water is highly dependent on algal or cyanobacterial species (Lee et al. 2017). It should be noted that a number of compounds are formed by both groups of algae, among them are DMS, 6-methyl-5-hepten-2-one, *b*-cyclocitral, *b*-ionone, 2,4,7-decatrienal. However, only cyanobacteria, but not eukaryotic algae, form DMDS, DMTS, isopropyl disulfide, MIB, geosmin, geraniol, nerol, ketone, and ionone derivatives. Since cyanobacteria and eukaryotic algae inhabit the same niches, such differences may have competitive advantage and adaptive value for life in the microalgae community.

11.3 Intracellular Localization and Release of the Cyanobacterial VOCs

What is known about VOCs localization in the cyanobacterial cells? This issue is presently not well studied. Since MIB and geosmin originate from monoterpene and sesquiterpene precursors, respectively, the association of these VOCs with synthesis of lipophilic pigment was suggested (Bentley and Meganathan 1981). Later it was noted that geosmin synthesis by *Anabaena circinalis* and *Oscillatoria brevis* (Kutz) was related to chlorophyll *a* (Chl *a*) and carotenoid biosynthesis (Naes et al. 1985, 1988; Bowmer et al. 1992). At the same time Wu and Juttner (1988a) observed separation of MIB and geosmin in cellular structures of cyanobacterium *O. tenuis* Agardh, including the photosynthetic thylakoid membranes. It was suggested that there are distinct places of synthesis for these compounds. Wu and Juttner (1988a, b) showed that geosmin can be found in cyanobacterial cells in two intracellular

fractions: one is dissolved in the aqueous cytosol and a second is bound to proteins. Note that the results of these studies were based just on chemical analyses. The first structural study (Bafford et al. 1993) was made possible by the availability of polyclonal antibodies that were synthesized by Chung et al. (1990) against a derivatized conjugate of MIB. As a result, a specific label was detected along photosynthetic membranes. Therefore, it was suggested an existence of possible connection of MIB synthesis and the pigment systems.

These two earthy-muddy smelling terpenoids, geosmin and MIB, are retained within the cyanobacteria cells tightly bound to thylakoids with minimal release during growth. The bulk production of these VOCs occurs during culture senescence, death, or during grazing (Durrer et al. 1999; Jüttner and Watson 2007). Oxidative stress is shown to result in the release of intracellular odorous metabolites, e.g. geosmin and MIB, with concentrations exceeding threshold odor values in the three cyanobacteria, *M. aeruginosa*, *Oscillatoria* sp., and *Lyngbya* sp. (Wert et al. 2014).

It should be noted that isopropylthiols are continuously excreted over the growth cycle by *M. aeruginosa*, whereas large amounts of β-cyclocitral are generated at cell damage by grazers or death by all *Microcystis* species via enzyme-mediated catalysis (Jüttner 1984; Jüttner et al. 2010).

11.4 Cyanobacterial VOCs Receptors

How can cyanobacteria perceive and sense different VOCs? It is hypothesized that some "receiving devices," e.g. specialized receptors for the perception of the volatiles, must exist in cells, because it is unlikely that the different compounds diffuse through the membranes. This area of research is a fascinating and practically unexplored.

Ethylene, a well-known plant hormone, binds to the ethylene receptors and the information is intracellularly transmitted (Lacey and Binder 2014). Mount and Chang (2002) reported about two genes (*all0182* and *alr4716*) in the genome of cyanobacterium *Anabaena* sp. strain PCC 7120 that encodes homologs of the ethylene hormone receptors of higher plants. Further analysis of the cyanobacterium *Synechocystis* sp. strain PCC 6803 (*slr1212*) helped to find a third homolog. Mount and Chang (2002) concluded that the ancestral progenitor of plant ethylene receptors is likely to be a cyanobacterial ethylene receptor. Lacey and Binder (2016) demonstrated that the cyanobacterium *Synechocystis* sp. PCC 6803 has a functional receptor for ethylene, *Synechocystis* Ethylene Response1 (SynEtr1). It is the first demonstration of a functional ethylene receptor in cyanobacteria. It was shown that SynEtr1 directly binds ethylene. Treatment of *Synechocystis* cells by ethylene or disruption of the *SynEtr1* gene affects several processes, including phototaxis, photosystem II levels, type IV pilus biosynthesis, biofilm formation, and spontaneous cell sedimentation. Obtained data suggest a model where SynEtr1 inhibits downstream signaling and ethylene inhibits SynEtr1. This is similar to the

inverse-agonist model of ethylene receptor signaling proposed for plants and suggests a conservation of structure and function that possibly originated over 1 Ga. Prior research showed that SynEtr1 also contains a light-responsive phytochrome-like domain (Narikawa et al. 2011; Song et al. 2011). Thus, SynEtr1 is apparently a bifunctional receptor that mediates responses to both light and ethylene (Lacey and Binder 2016).

11.5 Factors Affecting Cyanobacterial VOCs Production

Cyanobacteria synthesize and release wide spectrum of VOCs throughout growth in aquatic and terrestrial ecosystems (Möhren and Jüttner 1983; Evans 1994; Watson 2003; Höckelmann and Jüttner 2005; Achyuthan et al. 2017). VOCs synthesis and emission are coupled with cell energy status and thus is related to photosynthesis and pigment synthesis, and to environmental factors that modify these processes (Watson 2003). Among them are light, temperature, nutrients (phosphorus, iron, and nitrogen availability), salinity, aeration (mixing/turbulence) or static culture, phage infection, cyanobacterial population density, culture age (senescence or apoptosis) and the presence of grazers.

Light and Dark Conditions

The effect of light on the synthesis of various VOCs by cyanobacteria is different for various types of compounds. Cyanobacteria release isoprene depending on light conditions (Shaw et al. 2003), e.g. the isoprene emission rate from cyanobacterium *Prochlorococcus* increased with raising light intensity (Bonsang et al. 2010). Isoprene and bromomethane (CH_3Br) were produced by *Synechococcus* sp. (NIES-981) when Chl *a* increased in the early stage of the incubation period (5–15 days of incubation time, exponential phase), while CH_3Br was produced when Chl *a* was reduced in the late stage of the incubation period (30–40 days of incubation time, death phase) (Hiraiwa et al. 2014).

Geosmin production occurs at constant temperature of 20 °C and under different light regimes it correlates with Chl *a* in *O. brevis* (Kutz) (Naes et al. 1985). Similar results were obtained for *Anabaena circinalis* and planktonic and benthic *Oscillatoria* (Bowmer et al. 1992; Utkilen and Frøshaug 1992). Contrasting results were demonstrated in *A. circinalis* (Saadoun et al. 2001), an inverse relationship between Chl *a* and geosmin was shown. Temperature also had an apparent effect on geosmin production in *Lyngbya kuetzingii*, possibly by inhibiting Chl *a* synthesis, since highest geosmin concentration correlated with a low Chl *a* concentration at 10 °C (Zhang et al. 2009). Since in these and others studies different organisms and different growth conditions were used, it is difficult to draw a valuable conclusion about the correlation between Chl *a* and geosmin production.

Sulfur VOCs production was considerably lower under light conditions relative to dark incubations in cyanobacterial mats from a hot spring in Yellowstone National

Park (USA) (Zinder et al. 1977). Only traces of DMS were found but instead large amounts of methyl mercaptan and H_2S.

The influence of photooxidative conditions on the composition of VOCs released was studied by the cyanobacterium *M. aeruginosa* (Walsh et al. 1998). As cell numbers increase during with sunlight exposure in medium with low and high iron, the most abundant volatiles released are naphthalene, terpenoid compounds, aliphatic hydrocarbons (C15–C21), β-cyclocitral, and β-ionone. Exposure of cyanobacterium to sunlight at low iron concentrations decreased β-cyclocitral, heptadecane, β-ionone and the total VOCs concentrations were measured. It was concluded that exposure to both high iron concentrations and high light irradiance affected the VOCs composition since photooxidation results in intracellular changes that alter the volatile compound profile.

Phosphorus and Nitrogen Supplies

Lack of nutrients leads to increased synthesis of secondary metabolites by cyanobacteria. Many of them are toxic to phytoplankton and are used as allelopathic tools in the competition of cyanobacteria for more resources within their habitat.

VOC emissions from cyanobacteria *M. aeruginosa* under different phosphorus sources and concentrations were investigated (Ye et al. 2018). It was demonstrated that use of K_2HPO_4 in growth medium in comparison with $Na_4P_2O_7$, and $(NaPO_3)_6$ showed the largest increase in cell density of *M. aeruginosa* and higher number of different VOCs emission (26, 23, and 22 VOCs, respectively), including sulfocompounds, terpenoids, benzenes, hydrocarbons, alcohols, aldehydes, and esters. The absence of any phosphorus in the growth medium significantly promoted the VOC emission, and six additional compounds were detected: α-pinene, 1-phenyl-1-butanone, 1*H*-1-ethylidene-indene, 2,6,10-trimethyl-tetradecane, 2-ethylhexanal, and acetic acid 2-ethylhexyl ester. It was concluded that cyanobacteria release different VOCs using various phosphorus sources in eutrophicated waters, and the reduction of phosphorus amount promotes VOC emission (Ye et al. 2018).

The effects of complex phosphorus compounds on the VOC emission from cyanobacteria and their toxic effects on other algae were investigated. The VOCs from *M. aeruginosa* (Yang et al. 2018) and from *Microcystis flos-aquae* (Zuo et al. 2018) grown with different types and amount of phosphorus nutrients were analyzed, and VOC compounds affected the growth of green algae *Chlamydomonas reinhardtii*. Predictably, both cyanobacteria showed slow growth in medium with low phosphorus concentrations or in phosphorus-free medium, but they showed high VOC emission. Among the volatiles furans, sulfocompounds, terpenoids, benzenes, aldehydes, hydrocarbons, and esters were detected (Zuo et al. 2018). Cyanobacterium *M. flos-aquae* growing in phosphorus-free medium emitted two main terpenoids (eucalyptol and limonene) in the VOCs mixture. It was found that cell growth, photosynthetic pigment content, and photosynthetic abilities noticeably decreased in *C. reinhardtii* in the presence of these VOCs. It was concluded that multiple phosphorus nutrients in eutrophicated waters induce different VOC emissions from cyanobacteria, and phosphorus amount reduction caused by natural precipitation and algal massive growth results in more VOC emissions. These VOCs are toxic

(e.g., eucalyptol and limonene) and subsequently cyanobacteria become dominant species (Zuo et al. 2018). In addition, Dzialowski et al. (2009) found a negative relationship between geosmin amount and inorganic phosphorus concentration in reservoirs through field works in Kansas (USA).

Similar trends of VOC emission by cyanobacteria were observed in case of different nitrogen sources. For example, *M. aeruginosa* cells increased the emission amount of alcohols and β-cyclocitral upon nitrogen starvation (Hasegawa et al. 2012). Cyanobacterium *M. flos-aquae* cells released different VOC components and amounts under different nitrogen sources (Xu et al. 2017). The amount of VOC emission increased with reducing nitrogen concentration and the maximum emission was under nitrogen-free conditions. Moreover, under nitrogen starvation, *M. flos-aquae* VOCs showed significant inhibitory effects on *Chlorella vulgaris* cell growth with the reduction in photosynthetic activity of the green algae (Xu et al. 2017).

In the experimental work with selected cyanobacterial strains of *Spirulina*, *Anabaena*, and *Nostoc* genera originating from Serbia it was shown that addition of nitrogen to growth medium negatively affected the production of 2-methylisoborneol, while geosmin was not detected in any of the analyzed samples (Milovanović et al. 2015). Authors suggested that the manipulation of growth conditions might be a useful tool in reducing levels of some unwanted odor-causing components in water.

As outlined above, the VOC emission by cyanobacteria is under the control of many various factors. Bonsang et al. (2010) showed that isoprene is emitted from living phytoplankton cells at variable rates depending on the light intensity, cell volume, and carbon content of the plankton cells. In another study it was demonstrated that isoprene production was a function of light intensity and temperature in *Prochlorococcus*, e.g. it decreased by 84% after 48 h in darkness (Shaw et al. 2003). Moreover, isoprene production by *Prochlorococcus* had a temperature maximum at 23 °C, at which the photosynthetic rate was maximal. It was concluded that isoprene production rates per phytoplankton cell varied with cellular growth phase. Constant, maximum rates were achieved during balanced exponential growth, while decreasing rates were found during population senescence, and phage infections decreased isoprene production as well (Shaw et al. 2003).

11.6 VOCs of Cyanobacteria as Allelopathic Tools

Allelopathy

Allelopathy is a property of different organisms (microorganisms, fungi, plants, animals) to secrete chemical compounds that influence the development and growth of other organisms. According to Hans Molisch (1937), allelopathy covers biochemical positive and negative interactions between plants. Cyanobacteria and algae often involved in competition for space, light, and nutrients. The biosynthesis and release of allelopathically active compounds interfere with settlement and growth of

competitors (Lee 2008; Li and Li 2012). They have negative or positive biological effects on different organisms (Reigosa et al. 2006).

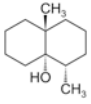
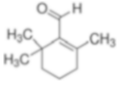
The appearance of allelopathic chemicals is common in cyanobacteria and algae communities (Gallucci and Paerl 1983; Smith and Doan 1999; Fink 2007; Dobretsov et al. 2010; Gross 2003). Growing populations of cyanobacteria are able to control their cell density and manage it depending on the surrounding conditions. To do this, they use various secondary metabolites. Cyanobacteria use biologically active compounds also to outcompete other species, but the specific mechanism of action of several secreted compounds is still not well characterized. Fundamental studies were performed on the cyanobacterial dominance in eutrophic lakes by Keating (1977, 1978). Allelopathy in pelagic environments depends on sufficient biosynthesis and excretion of allelochemicals and their effective distribution in waters that can permit to reach other species at effective concentrations. In lakes, allelopathic compounds play remarkable roles in the regulation of bloom formation and development (Keating 1977; Vardi et al. 2002). In rivers, excreted algal secondary metabolites may be carried away by the current, therefore allelopathy may be less significant in planktonic communities. Dilution is the major problem. Therefore, to study allelopathic chemicals released and their mode of biological action on targets, laboratory-controlled experiments were performed (Flores and Wolk 1986; Gromov et al. 1991; Doan et al. 2001; Suikkanen et al. 2004; Chang et al. 2012; Śliwińska and Latała 2012; Złoch et al. 2018). In aquatic environments allelopathic interactions are most relevant between organisms living adjacent to each other in littoral or benthic zones.

Many cyanobacteria and groups of algae produce diverse VOCs (Ali 2004; Fink 2007), while more studies were performed with VOCs of eukaryotic microalgae (Zuo et al. 2012; Vanelslander et al. 2012; Durme et al. 2013). The ecological role of cyanobacterial VOCs is largely unexplored. It was hypothesized that the VOC production is very important for cyanobacteria living in very changeable aquatic surrounding (Zuo 2019). Among cyanobacterial VOCs, some chemicals have been discovered as the allelopathic compounds. They are eucalyptol, geosmin, limonene, geranylacetone, β-cyclocitral, α-ionone, and β-ionone. These chemicals can benefit the producers' growth in competition for limited nutrients (Ikawa et al. 2001) (Table 11.1). Cyanobacteria use allelochemicals in their defense against herbivores. Since the biosynthesis of chemicals is costly for cyanobacteria it seems energetically more beneficial for cells to biosynthesize the metabolites upon stress, e.g. by different abiotic and biotic factors (light, temperature, pH, availability of nutrients, grazers, and competitors presence).

Inhibition of Competitors by Cyanobacterial VOCs

To test whether they are allelopathically active, synthetic compounds of various VOCs commonly found in cyanobacteria and algae were applied (geosmin, 2-MIB, β-ionone, β-cyclocitral) in agar diffusion assays, and they were shown to inhibit growth of the eukaryotic green alga *Chlorella pyrenoidosa* (Ikawa et al. 2001). Other VOCs (monoterpene alcohols, straight chain alcohols and aldehydes, straight chain fatty acids (C7–C12)) also repress growth of algae. It was concluded that these

Table 11.1 Allelopathic functions of cyanobacterial VOCs

VOC	Formula	Strain producer	Target	Type of allelopathy	References
Geosmin	$C_{12}H_{22}O$ 	*Anabaena Oscillatoria variabilis*	*Chlorella pyrenoidosa*	Inhibition of competitors	Ikawa et al. (2001)
		Kamptonema sp. PCC 6506	*Aedes aegypti* mosquitoes	Attraction	Melo et al. (2019)
2-MIB	$C_{11}H_{20}O$ 	*Anabaena Oscillatoria variabilis*	*Chlorella pyrenoidosa*	Inhibition of competitors	Ikawa et al. (2001)
β-Ionone	$C_{13}H_{20}O$ 	*Tychonema* sp.	*Chlorella pyrenoidosa*	Inhibition of competitors	Ikawa et al. (2001)
			Microcystis M. aeruginosa NIES-843	Anti-cyanobacterial	Harada et al. (2009) Shao et al. (2011)
			Nematodes	Repellent	Höckelmann et al. (2004)
β-Cyclocitral	$C_{10}H_{16}O$ 	*Microcystis aeruginosa* PCC 7005	*Chlorella pyrenoidosa*	Inhibition of competitors	Ikawa et al. (2001)
		Microcystis aeruginosa PCC 7820	*Nitzschia palea*		Chang et al. (2011)
			Daphnia magna	Grazers inhibition	Jüttner et al. (2010) Ozaki et al. (2008)
		Dolichospermum spp. *Microcystis*		Anti-cyanobacterial	Arii et al. (2015, 2018)
L-Limonene	$C_{10}H_{16}$ 	*Microcystis flos-aquae Microcystis aeruginosa*	*Chlorella vulgaris Chlamydomonas reinhardtii*	Inhibition of competitors	Zhao et al. (2016) Xu et al. (2017) Zuo et al. (2018) Yang et al. (2018)

(continued)

Table 11.1 (continued)

VOC	Formula	Strain producer	Target	Type of allelopathy	References
Eucalyptol	$C_{10}H_{18}O$ 	*Microcystis flos-aquae* *Microcystis aeruginosa*	*Chlorella vulgaris* *Chlamydomonas reinhardtii*	Inhibition of competitors	Zhao et al. (2016) Xu et al. (2017) Zuo et al. (2018) Yang et al. (2018)
Aldehydes		*Anabaena* sp. *Nostoc* sp.	*Chlorella pyrenoidosa*	Inhibition of competitors	Möhren and Jüttner (1983) Ikawa et al. (2001)
Ketones		*Anabaena* sp. *Nostoc* sp. *Pseudomonas* and *Serratia* strains	*Synechococcus* sp. PCC 7942	Inhibition of competitors	Möhren and Jüttner (1983) Popova et al. (2014); Voronova et al. (2019)
VOCs mixtures					
2-Butanone	C_4H_8O	*Calothrix* sp., *Calothrix parietina*, *Plectonema* sp. Natural biofilms and epilithic cyanobacterial biofilms of Lake Zurich	NematodesBursilla *monhystera* (Rhabditidae) *Caenorhabditis elegans* (Rhabditidae)	Attraction	Höckelmann et al. (2004)
3-Methyl-3-buten-1-ol	$C_5H_{12}O$				
2-Pentanol	$C_5H_8O_2$				
2,3-Pentandione	$C_6H_{10}O_2$				
2,3-Hexandione	$C_6H_{12}O$				
2-Hydroxy-3-hexanone 6-Methyl-5-hepten-2-one and others	$C_8H_{14}O$				
n-Pentadecanol	$CH_3(CH_2)_{13}CH_2OH$	*Chroococcus, Phormidium, Leptolyngbya, Spirulina*, and *Lyngbya*	Mosquitoes	Attraction	Rejmankova et al. (2000)

VOCs might act as allelopathic agents towards the phytoplankton (Ikawa et al. 2001).

M. flos-aquae produced toxic VOCs that inhibit photosynthesis and growth of *C. vulgaris* (Xu et al. 2017). Among many produced VOCs, limonene and eucalyptol were the major toxic compounds. Herewith, *C. vulgaris* cell division, Chl *a* and Chl *b* content were reduced significantly in presence of these VOCs, however, only in the nitrogen-free medium. The similar harmful effects of the VOCs from *M. aeruginosa* (Yang et al. 2018) and *M. flos-aquae* (Zuo et al. 2018) on *C. reinhardtii* growth were shown. Moreover, *M. flos-aquae* emitted limonene and eucalyptol in the absence of phosphorus into the growth medium. These VOCs suppressed the *C. reinhardtii* growth remarkably (Zuo et al. 2018). The effects of eucalyptol and limonene on algal photosynthesis were investigated in more detail and it was shown that these VOCs reduce the concentration of PSII reaction centers and inhibit the quantum production and electron transport in PSII (Zhao et al. 2016). Eucalyptol and limonene promoted dissipation of the absorbed light energy as heat. It was concluded that these terpenoids are allelopathic compounds of cyanobacteria by inducing degradation of photosynthetic pigments and reducing photosynthetic abilities in algae (Zhao et al. 2016). Recently it was discovered that terpenoids linalool and α-terpineol induced apoptosis in *C. reinhardtii* (Chen et al. 2019).

In summary, in nutrient limiting conditions cyanobacteria are able to increase VOCs production and some of the compounds are toxic for competitors. These allelochemicals provide an advantage to cyanobacteria in the fight for resources.

In addition, the effects of various concentrations of β-cyclocitral on the cells of diatom, *Nitzschia palea*, and cyanobacteria *M. aeruginosa* PCC 7005 and *M. aeruginosa* PCC 7820, were studied (Chang et al. 2011). It was discovered that diatoms are much more sensitive to β-cyclocitral, although dosage and contact time need to be determined to control microalgal growth in natural waters.

Dependence of Cyanobacterial Defense Mode on Grazer Pressure

The greatest loss of algal biomass occurs during herbivorous and predatory invertebrate grazing on algae and cyanobacteria. Algae use constitutive or inducible chemical defense mechanisms for protection (Lee 2008). During constitutive defense the chemicals involved in defense are present in cells all the time, while during an inducible defense the chemicals that function in defense are only produced when the microalgae is under pressure from a grazer. The latter is more economical for the microalgae cells. β-cyclocitral is often present in eutrophic waters and is a well-known airborne malodor of drinking water. It derives from the catalytic breakdown of β-carotene, and evidence indicates that it is produced by the activation of a specific carotene oxygenase by all species of the bloom-forming *Microcystis*. β-cyclocitral could be a grazer defense signal, for example, against on *Daphnia magna* (Jüttner et al. 2010). It was found that in living cyanobacterial cells β-cyclocitral was detected at very low concentrations or even undetectable. However, after cell damage by grazers the enzyme activity of carotene oxygenase was rapidly activated producing large amounts of β-cyclocitral (2.2 mM). Treatment of *Daphnia magna* with high amounts of β-cyclocitral caused a marked increase in its

swimming speed. The minimum β-cyclocitral concentration, which causes a significant response in the grazer, was 750 nM β-cyclocitral. Under natural conditions, disrupted cyanobacterial cells emitting β-cyclocitral lead to the development of a stable zone around the *Microcystis* colonies. Thus, this VOC is acting as a potent repellent and an indicative signal of "bad quality food" for grazers (Jüttner et al. 2010).

Anti-cyanobacterial Compounds

Different VOCs with an anti-cyanobacterial activity have been isolated and characterized, i.e. 3-methyl-1-butanol (Wright et al. 1991), β-cyclocitral, β-ionone, and geosmin (Ikawa et al. 2001). However, there are only a few studies on the mode of action of these compounds.

The anti-cyanobacterial activities of the volatile compounds such as *b*-cyclocitral, 3-methyl-1-butanol, and 2-decanone were studied by Ozaki and colleagues (Ozaki et al. 2008).

3-Methyl-1-butanol had only a weak lytic activity at a concentration of 6.5 mM, but showed a lysis activity at tenfold higher concentration. In contrast, *b*-cyclocitral and 2-decanone clearly affected Chl *a* concentration in the cells. This occurred rapidly after the addition, and the green color of the cyanobacteria disappeared within 7 h, and was completely gone within a day. 2-decanone caused color change from green to yellow-green and *b*-cyclocitral caused a characteristic color change from green to blue. This color change is similar to the phenomenon observed when a suddenly growth of cyanobacteria declined in a natural environment, which may be due to autolysis caused by *b*-cyclocitral production. In other studies (Arii et al. 2015, 2018) it was shown that cyanobacterial lysis with the release of phycocyanin was caused by the high density of cyanobacteria; the latter was accompanied by β-cyclocitral release. In the respective study the "graduated cylinder method" as a concentration technique was successfully applied where the concentrated *Dolichospermum* spp. and *Microcystis* gradually lysed. During the experimental concentration process the β-cyclocitral and its oxidation products were detected. Due to oxidation of β-cyclocitral corresponding carboxylic acid was formed and pH of the water decreased (to pH 6), which led to induced cyanobacterial cell lysis accompanied with phycocyanin release. This is a way of adaptive regulation of cyanobacterial phytoplankton cell density.

The terpenoid, β-ionone, is widely distributed in algae and higher plants (Zuo 2019). It derives from β-carotene which is cleaved through carotenoid cleavage of dioxygenases (Simkin et al. 2004). β-ionone causes cellular lysis and a decrease of Chl *a* content in *Microcystis* (Harada et al. 2009). Shao et al. (2011) explored the possible targets for β-ionone toxicity on the photosynthetic apparatus of *M. aeruginosa*. Several different parameters were investigated. Under β-ionone stress the Chl *a* fluorescence transients and transcript expressions of key genes encoding the photosynthetic system components of *M. aeruginosa* NIES-843 were studied. It was demonstrated that β-ionone leads to a decrease in pigment content of cyanobacterial cells. In addition, carotenoids were more sensitive to β-ionone stress than Chl *a*. The expression of *psbA* and *psbO* genes was down-regulated at

22.5 mg/L β-ionone. Ultrastructural changes indicated that the thylakoids were distorted, and the thylakoid membrane stacks began to collapse when *M. aeruginosa* NIES-843 was exposed to β-ionone at concentrations of 22.5 and 33.75 mg/L. The results indicated that the reaction center of PS II and the electron transport at the acceptor side of PS II are the targets of β-ionone and are responsible for the toxicity in *M. aeruginosa* NIES-843.

Cyanobacterial VOCs as Attractants and Repellents for Animals

VOCs are important infochemicals in biofilms of benthic microalgae. VOCs emitted by mat-forming cyanobacteria can serve for nematodes, insects, and possibly other organisms as habitat finding notes (Fink et al. 2006). Similar to findings from land plant–herbivore interactions, Durrer et al. (1999) have demonstrated that grazing by herbivorous *Cladocera* on planktonic cyanobacteria adduces to the emission of large amounts of VOCs which was shown in field and laboratory experiments. Also, when snails or other invertebrates graze on an algal biofilms, algal cells will rupture and release VOCs. Other snails are then able to detect this food patch.

Some aquatic insects (Evans 1982) and free-living nematodes (Höckelmann et al. 2004) use cyanobacterial VOCs as habitat finding tags. Invertebrates inhabit algal mats that represent the complex mixture of different cyanobacteria, many heterotrophic bacteria, various algae and detritus. Since cyanobacteria are abundant source of VOCs (Jüttner 1987), it is possible that aquatic organisms can utilize such VOCs as markers of high local presence of cyanobacteria. In the work of Rejmankova et al. (2000) a "tendency" for an enhanced oviposition rate of mosquitoes in containers that contained the *n*-pentadecanol was demonstrated. Cyanobacterial mats represent the mosquitoes' preferred oviposition substrate. Cyanobacteria emit VOCs that are structurally similar to *n*-pentadecanol. However, there is a need for further more detailed studies of the cyanobacterial VOCs impact on terrestrial and semi-aquatic insects. Recently it was shown that geosmin-producing cyanobacteria confer *Aedes aegypti* oviposition and larval attraction (Melo et al. 2019). The yellow fever mosquito *A. aegypti* detects geosmin with a high degree of sensitivity. *A. aegypti* larvae find geosmin attractive, as well as geosmin-producing cyanobacteria *Kamptonema* sp. (*Oscillatoria*) PCC 6506. This strain produces the neurotoxin anatoxin-*a* (or Very Fast Death Factor, VFDF) and the cytotoxin cylindrospermopsin (Mazmouz et al. 2010). While the species producing geosmin will attract mosquitoes, others cyanobacterial strains will show strong larvicidal effect. It is presently not understood how mosquito larvae are tolerant to cyanobacterial toxins and/or mosquitoes might separate harmful cyanobacteria from harmless (Melo et al. 2019). Considering that many natural or recombinant cyanobacteria can be toxic to mosquitoes (Thiery et al. 1991; Kiviranta et al. 1993; Singh et al. 2003; Boussiba et al. 2000; Mekhlif and Khudhair 2016), different combinations of various strains of cyanobacteria could be applied in mosquito control. Nematodes can smell and taste cyanobacterial VOCs (Höckelmann et al. 2004). The chemotaxis responses of the freshwater nematode *Bursilla monhystera* (Rhabditidae) and the terrestrial model organism *Caenorhabditis elegans* (Rhabditidae) to VOCs emitted by cyanobacterial biofilms were studied by using

two epilithic cyanobacterial biofilms of Lake Zurich. Artificial, axenic biofilms of *Calothrix* sp., *C. parietina*, and *Plectonema* sp. were investigated and it was found that the epilithic biofilms and the axenic cyanobacteria had many common VOCs. It is curious how different nematodes differ in their bias towards VOCs emitted by different cyanobacteria. *B. monhystera* was significantly attracted to *C. parietina* and *Plectonema* sp. but not to *Calothrix* sp. In contrast, terrestrial *C. elegans* was not attracted to any of these cyanobacterial biofilms when a variety of single odor compounds were offered to both animal species. *B. monhystera* did not like any of the individual VOCs tested, β-ionone even repelled this species. Aquatic nematodes are attracted to cyanobacterial biofilms using multicomponent odor as chemical cues (Höckelmann et al. 2004). Remarkably, VOCs released from benthic cyanobacteria and algae can be utilized as food and habitat finding tags by freshwater gastropods and nematodes (Fink 2007).

It is a question, whether other animal species that depend on cyanobacterial mats are able to detect and utilize the different VOCs emitted by periphytic cyanobacteria (Izaguirre and Taylor 1995; Watson and Ridal 2004). Considering the widespread ability of terrestrial insects to perceive VOCs, it is reasonable to hypothesize that the perceptions of VOCs from aquatic sources by terrestrial and semi-aquatic insects (Evans 1982; Rejmankova et al. 2000) participate can contribute to the regulation of food-web interactions between aquatic and terrestrial ecosystems. Further research on VOC-mediated behavioral strategies of insects that switch between aquatic and terrestrial habitats during their life-cycle could therefore help to find and understand the factors mediating the connections between the two ecosystems. The importance of microbial secondary metabolites in insect life is just beginning to receive appropriate attention. Nowadays, advances in modern analytical techniques will permit researchers to go deeper into the complexity of microbe–insect associations, and to study in details the influence of microbial metabolic activity on the behavior of higher organisms (Davis et al. 2013).

Thus, the volatiles can increase the resistance of cyanobacteria to different abiotic stresses; they can protect cyanobacterial cells against grazers and can transfer information between cyanobacteria and other organisms. Under stress conditions the VOCs synthesized and released from the cyanobacteria serve as infochemicals to other cells, preparing them to resist for the approaching stress (Fig. 11.1).

11.7 Conclusions and Perspectives

Cyanobacteria produce a large amount of the different VOCs. Many of them are biosynthesized by these ancient photoautotrophic prokaryotes under different stress conditions. Some VOCs are produced during nutrient deficiency in the growth environment and these compounds suppress cyanobacterial competitors for limiting resources. VOCs of cyanobacteria can affect a behavior of grazers, thus acting as a powerful repellent and a signal of “low quality food” to grazers. These compounds control the growth and density of cyanobacterial populations acting as

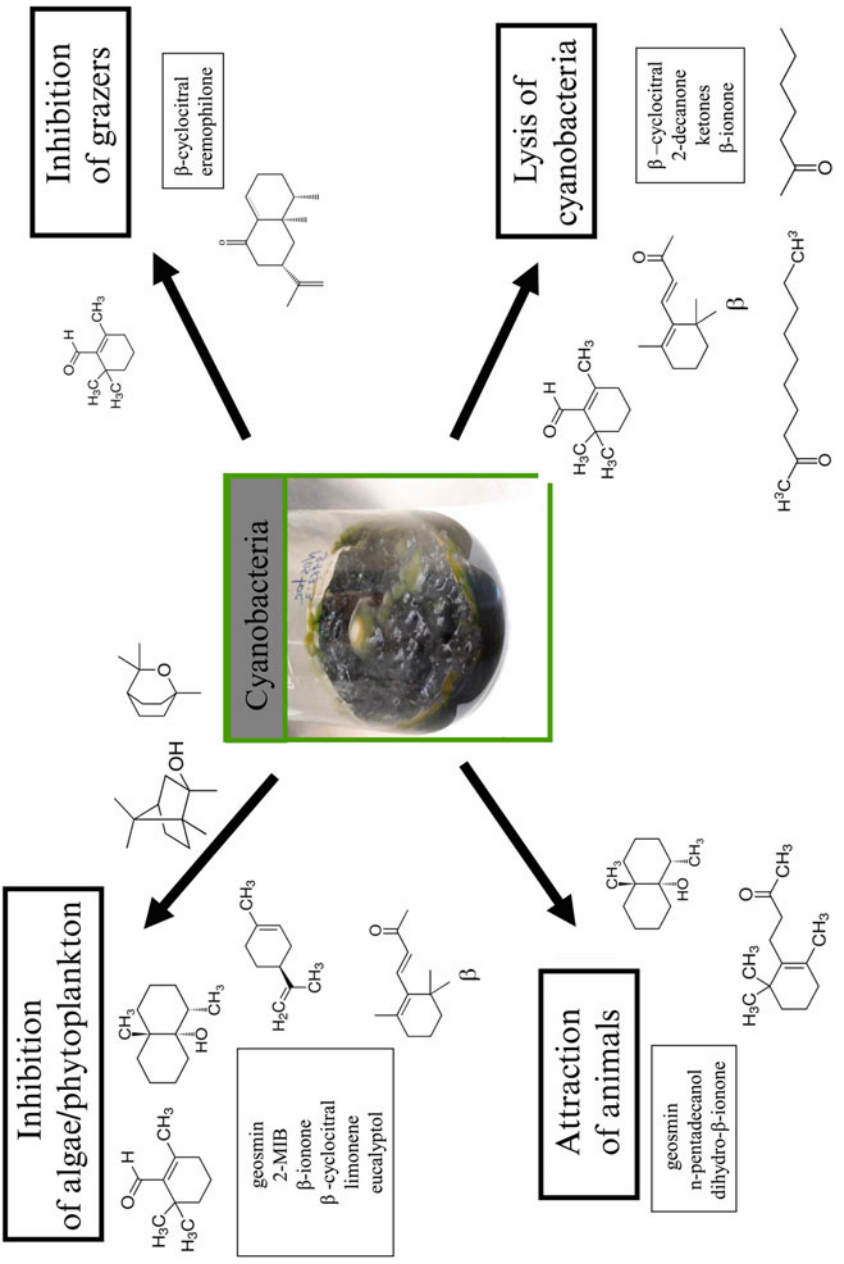


Fig. 11.1 Schema representing the main biological activities of cyanobacterial VOCs. Photo of a two-year-old laboratory culture of *Nostoc* sp. 8963 (photo provided by author)

"infochemicals." Cyanobacterial VOCs attract invertebrate animals such as mosquitoes and nematodes and may serve as nutrition. Consequently, cyanobacterial VOCs have important functions in different ecosystems.

There are still a large number of intriguing questions that need to be clarified; including the temporal and spatial analysis of cell-bound and dissolved VOCs; the molecular regulation of their production and perception; the processes underlying the resistance of the cyanobacteria themselves to the high concentrations of VOCs; the molecular signals that induce the synthesis of VOCs in cells under certain conditions; the major molecular, biochemical, and physiological traits which determine which strains are low and high odor producers; the molecular mechanisms which trigger and regulate their extracellular release; the relation (correlation) between the VOC biosynthesis and cyanotoxin production; the VOC effects on DNA stability, genotoxicity, and gene expression; the physiological and ecological roles of these compounds (i.e., chemical ecology), and many others. Application of modern tools of metabolomics (Bedia et al. 2018), transcriptomics (Nagashima et al. 2019), genomics, and bioinformatics, as well as mutagenesis and gene cloning in the future, will provide deep insight into mechanisms and regulation of biological activity of many cyanobacterial volatile organic compounds.

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Chapter 12
Sampling, Detection, Identification, and Analysis of Bacterial Volatile Organic Compounds (VOCs)

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Abstract Bacterial volatile organic compounds (VOCs) are signaling molecules mediating diverse inter- and intra-kingdom communication processes. Investigations to characterize bacterial VOCs according to their chemical nature, production, and emission into the environment need appropriate methods of sampling, detection, identification, and data analysis. Each of these individual steps requires careful examination of the biological question and its technical implementation. In the following chapter, we focus on various methods, which are currently used to sample, detect, identify, and analyze bacterial VOCs highlighting their advantages and/or limitations.

Keywords Bacteria · Volatile organic compounds · SPME · GC/MS · Direct injection mass spectrometry · eNose · Multivariate data analysis

12.1 Introduction

Bacterial volatile organic compounds (VOCs) are signaling molecules mediating diverse inter- and intra-kingdom communication processes (Ryu et al. 2003; Wenke et al. 2012; Effmert et al. 2012; Kim et al. 2013; Lemfack et al. 2016; Schulz-Bohm et al. 2017a, b, etc.). So far, ca. 1000 bacterial VOCs have been reported from bacteria belonging to diverse families including *Pseudomonas*, *Bacillus*, *Serratia*,

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etc. (Lemfack et al. 2018). Bacteria have, indeed, the machinery to produce a myriad of volatiles, including short-chain aliphatic aldehydes, esters, alcohols, organic acids, ethers, ketones, sulfur compounds, nitrogen compounds, and hydrocarbons (Schulz and Dickschat 2007; Lemfack et al. 2018; Kai 2020).

Investigations to characterize bacterial VOCs according to their chemical nature, production, and emission into the environment need appropriate methods of sampling, detection, identification, and data analysis. Each of these individual steps requires careful examination of the biological question and its technical implementation. (1) When isolating bacteria from nature as one part of the sampling process, for instance, it has to be considered that different habitats can carry varying specific microbial footprints (Bulgarelli et al. 2013). (2) Abiotic and biotic conditions (nutrients, temperature, oxygen availability, inter- or intraspecific interaction, etc.) that bacteria are exposed to during cultivation influence the quality and quantity of produced VOCs (Kai et al. 2009, 2016; Blom et al. 2011; Weise et al. 2012; Somerville and Proctor 2013; Kai 2020). (3) Application of different VOC trapping techniques during VOC sampling can influence the range of chemical classes of VOCs that are collected (Kai et al. 2007, 2010; Kai 2020). (4) During VOC detection an improvement of the chemical resolution by chromatographic separation of VOCs from the VOC mixture is on costs of the temporal resolution. (5) Described bacterial VOCs might be wrongly identified due to inadequate compound identification (Schulz and Dickschat 2007; Kai 2020). (6) And finally, data evaluation needs appropriate multivariate data analysis tools to draw adequate trend-setting conclusions.

All these issues, we try to address in the following chapter in which we focus on various methods, which are currently used to sample, detect, identify, and analyze bacterial VOCs highlighting their advantages and/or limitations.

12.2 Capturing Bacterial VOCs: Methods Used for Collection

12.2.1 Sampling of Bacterial VOCs

The first and most challenging question for any researchers, who step into the field is how to sample VOCs. The sampling process is crucial for analysis of volatiles, since it affects all further outcomes including data interpretation and postulation of hypotheses. Basically, there are two major approaches to sample bacterial VOCs, the in situ sampling or the isolation of the bacteria from their natural habitat and subsequent in vitro VOC sampling. The latter is commonly performed in laboratories due to its easy setup and less interference with background VOCs, albeit, in situ sampling mirrors the total content of VOCs occurring in a natural habitat. The linking of grassland soil VOCs to bacteria and plants showed that the proportion

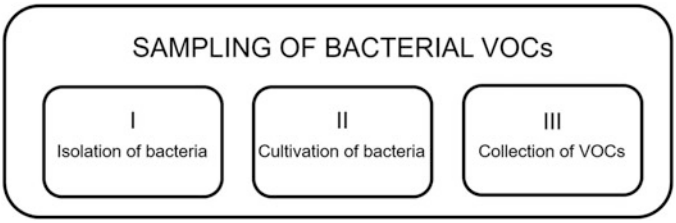

Fig. 12.1 Workflow of critical steps in in vitro bacterial VOC sampling

of in situ detected VOCs correlated with single bacterial taxa was higher than for plants (Schenkel et al. 2019); however, the major disadvantage of in situ sampling is that volatiles cannot be assigned to the host (animal, plant, fungus) or to a single bacterial species. Systems that try to assign VOCs to bacteria by using proper controls are still in its infancy (Kai et al. 2016). Consequently, in vitro analyses of isolated bacteria are still required. The individual VOC profiles can in turn be compared with in situ VOC profiles to assign the causal bacterial producer. Sampling for in vitro VOC investigations includes: (1) the isolation of bacteria from their natural habitat (2), the artificial cultivation of these isolated bacteria in liquid cultures or on solid medium, and finally (3) the collection of their emitted volatiles (Fig. 12.1).

12.2.1.1 Isolation of Bacteria

Bacteria cover almost every spot on Earth. They are part of diverse and dynamic communities not only with other bacteria, but also with fungi and protists. These communities often colonize tissues and organs of plants and animals and are referred to as their microbiomes (Ding and Schloss 2014; Bai et al. 2015). Microbiomes play an essential role for plant and animal health, for instance, by producing volatile and non-volatile metabolites with signaling or antagonistic properties that help the host to cope with their environment.

In the past, about 600 bacterial species were isolated and analyzed for their VOCs (Lemfack et al. 2018). Presumed that the Earth is home of 10^{10}–10^{12} bacterial species (Locey and Lennon 2016), this clearly indicates that this number represents only the "tip of the iceberg." For plant microbiomes, for instance, it was noted that VOCs of only 10% of the bacterial genera found in known plant microbiomes are described so far (Piechulla et al. 2017).

It has further to be considered that microbiomes are highly specific for the different host organisms, e.g., plants and animals. Within the plant kingdom, the microbiome is further varying between different plants, between different organs (flower, leaf, root, etc.) but also changes according to developmental stages (Bulgarelli et al. 2013). This highly specific distribution of bacteria is connected with adapted metabolism fitting to the requirement of the respective ecological niche. It can be speculated that the capacity to produce VOCs is as adapted as

their general metabolism leading to variation in VOC profiles between different bacterial species. Bacteria analyzed so far for their emitted VOCs were obtained from at least eight distinct habitats including animals, humans, living plants (endophytes, leaf bacteria, rhizobacteria, and floral bacteria), dead plants (waste and litter), food sources, marine environment, bulk soil, and fresh water (Leff and Fierer 2008; D'Alessandro et al. 2014; Piechulla and Lemfack 2016; Helletsgruber et al. 2017); however, more systematic investigations in future considering the plant (animal), organ, or the developmental stage during bacterial isolation will strongly deepen the knowledge of VOC production in bacteria and moreover its regulation.

12.2.1.2 Cultivation of Bacteria During VOC Collection

Due to the high complexity of natural conditions including chemical parameter, environmental factors, nutritional conditions, and biotic interactions, investigations regarding the production and role of bacterial volatiles in nature remain quite challenging. In principle, most of the current knowledge was acquired using artificial culture conditions in in vitro test systems. Culture conditions such as nutrients, temperature, pH-value, and oxygen availability determine bacterial growth and development. Research has shown that these conditions can also influence the quality and quantity of the production of bacterial VOCs (Kai et al. 2009; Blom et al. 2011; Weise et al. 2012). Changes of nutrients, for instance sugars or amino acids, can lead to drastic shifts in VOC profiles (Kai et al. 2009; Blom et al. 2011; Weise et al. 2012). Therefore, the cultivation of bacteria should always be adapted to the biological question. In the last years, cultivation under nutrient rich conditions (complex medium: nutrient broth, lysogeny broth, tryptic soy broth, etc.) on solid medium in Petri dishes or in liquid medium in modified Erlenmeyer flasks showed the great capacity of bacteria to emit a broad range of VOCs. Since these nutrient rich media emit a high level of background VOCs, precise background subtraction is necessary to characterize bacterial VOCs (Schulz and Dickschat 2007; Farag et al. 2013; Kai 2020). Murashige–Skoog (MS) and other minimal media produce lower background signals than complex media; however, the effects of media on bacterial growth and development have also to be considered when choosing suitable nutrient conditions. The rhizobacterium *S. plymuthica* 4Rx13, for instance, showed 7–60 fold lower cell numbers, when growing on a minimal medium compared to the growth on complex medium (Kai and Piechulla 2018). In order to confirm to what extend VOCs are produced in situ, in future, cultivation parameter should finally be adjusted to conditions of the natural habitat. Plants, for instance, exude metabolites depending on species, age, and developmental stage (Bulgarelli et al. 2013). First experiments using plant root exudates as nutrients to simulate the soil environment along the rhizosphere are very promising in approaching natural conditions (Schulz-Bohm et al. 2015; Kai and Piechulla 2018). Temperature should also be adapted to the habitat in which the bacteria are naturally occurring, e.g., 37 °C for human associated bacteria. In the same way, the oxygen availability and the pH-value should fit with the natural habitat. For example, plant growth promoting

rhizobacteria emitted different VOCs when grown in presence of oxygen versus limited oxygen partial pressure (Farag et al. 2006). Bacterial cultures face also varying oxygen availabilities when growing on solid agar plates compared to cultures in liquid medium (Somerville and Proctor 2013).

Furthermore, most of the existing investigations studied the emission patterns of mono-cultivated pure bacterial cultures neglecting the biotic interactions that usually occur with their "neighbors" within the microbiome, but also with their host. Increasing evidence emerges that co-culturing of bacterial species affects microbial metabolism (Jones and Wang 2018), which in turn might influence the aroma composition. First approaches considering the interactive traits, indeed, showed that interaction of bacteria significantly influences the bacterial VOC emission (Schulz-Bohm et al. 2015; Hol et al. 2015; Tyc et al. 2015, 2017; Schmidt et al. 2017; Kai and Piechulla 2018). Furthermore, bacteria can individually release precursor VOCs, which are able to react in the bacterial headspace to form new VOCs that are not detected in the headspace of bacteria growing physically isolated from each other (Kai et al. 2018). More systematic investigations in future considering the characteristics of the natural habitat will allow gaining further knowledge of the significance of bacterial VOCs in nature.

12.2.1.3 Collection of Bacterial VOCs

Once the questions regarding cultivation conditions have been clarified, an effective and powerful system for collecting the VOCs must be applied. VOCs can be collected either using direct solvent or adsorption techniques, or trapped from the headspace of the cultivated bacteria. Only the use of headspace systems ensures that only VOCs emitted by bacteria are captured. Consequently, they are the preferred experimental approaches to be used. Headspace VOC collection can be categorized based on its setup to static and dynamic headspace systems. These systems fundamentally differ in regard to environmental factors influencing the bacterial culture, the type of VOC extraction, and the time frame of analysis.

Static Headspace Systems (HS)

The sampling of headspace VOCs of a bacterial culture without exchanging air between the interior air of the culture and the outdoor space characterizes a static headspace (HS) system. Due to their simple application, they are the favored experimental setups. VOCs can be profiled at certain different time points during growth and related to the bacterial growth stage. The main advantage of static HS systems is the enrichment of VOCs in the analysis chamber in which the bacterial culture is located. On the other side, the enrichment of bacterial metabolites can reach highly non-physiological/non-natural levels in the chamber that can subsequently modify the metabolism of the tested bacterial isolate (Kai and Piechulla 2009, 2010; Kai et al. 2016).

One method to collect the VOCs from the static bacterial headspace is *solid phase micro extraction (SPME)*. SPME was established by Pawliszyn in 1990 and

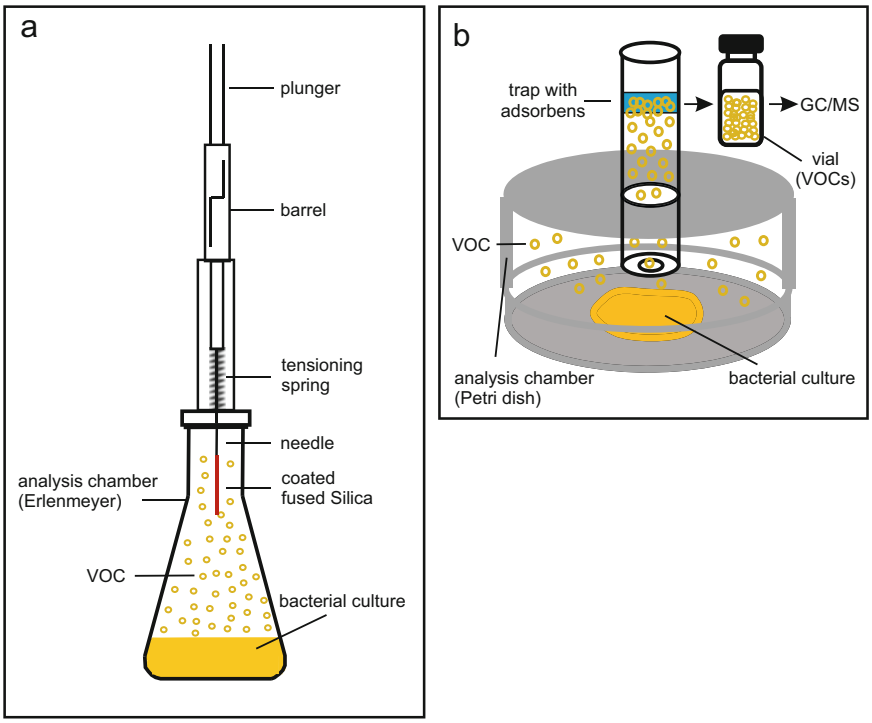

Fig. 12.2 VOC collection in static headspace systems (**a**) using SPME from a bacterial liquid culture, (**b**) using an adsorption material containing trap from a bacterial solid culture

represents a simple and cheap method to fast and efficiently extract VOCs (Arthur and Pawliszyn 1990). SPME is the dominating technique in the field and has been widely used in a large number of in vitro studies. VOCs can be trapped from the headspace of solid or liquid bacterial cultures (Fig. 12.2a). For sampling VOCs, a fused silica fiber coated with adsorption material (polydimethylsiloxane, carboxen, polyacrylate, and divinylbenzene solely or in combination) is exposed to the headspace, and trapped VOCs are subsequently thermally desorbed and analyzed in a gas chromatograph/mass spectrometer (GC/MS, see Sect. 12.3.1).

To obtain a comprehensive profile of bacterial VOCs, it is recommended to test different fibers before application. The selection of the adsorption fiber (thickness and coating) dictates the VOC classes that are detected. Thicker coatings are recommended for better detection of volatile compounds, while semi-volatile compounds are better retained using a thin fiber. The coating polyacrylate is used to extract strongly polar compounds, whereas polydimethylsiloxane is recommended to extract non-polar VOCs. Due to the selective adsorption of the coatings, the SPME technique has limitations regarding the absolute quantification of VOCs. Another limiting factor is that the extracted amount of VOCs using SPME is only sufficient for GC/MS analysis. Structure elucidation of unknown VOCs detected

with SPME needs further enrichment processes using either dynamic HS systems or alternative passive HS systems. In these alternative passive HS systems, *traps* containing adsorption material (see below) are used for quantification and enrichment of VOCs. These traps are, for instance, connected to the headspace of modified Petri dishes (Fig. 12.2b). Thereby, bacterial VOCs can passively diffuse from the chamber of the Petri dish to the adsorption material filled in a stainless steel—or glass tube that is connected to the lid of the Petri dish. After incubation for a certain time, VOCs are solvent-extracted from the adsorbent containing trap. The resulting extract is analyzed using GC/MS (see Sect. 12.3.1).

Dynamic Headspace (HS) Systems

The sampling of VOCs by streaming a continuous airflow over a bacterial culture changes the system towards a dynamic headspace (HS) system. The airflow entrains the headspace bacterial VOCs, which are subsequently trapped on adsorption material (SuperQ, Porapack, Tenax, or charcoal). This adsorption material is embedded in steel—or glass tubes, hereinafter referred to as *traps*, which are connected to the exit of a vessel containing a bacterial culture. There are three types of dynamic HS systems: open systems, closed systems, and closed loop systems (closed loop strip apparatus, CLSA).

In all three types charcoal purified, sterile air streams into an analysis chamber. Humidification of this inlet air by flow through a saturated salt solution can help to prevent dryness of the culture due to water evaporation. As analysis chamber either a vessel containing an inoculated Petri dish can be used for solid cultures, or for liquid cultures an Erlenmeyer flask. In both cases, the analysis chambers need an in- and outlet for airflow (Fig. 12.3). The airflow is generated by pumps. In *open systems*, there are two pumps. One pump is pushing air into the analysis chamber, while a second pump is pulling half of the influx air out directly on the adsorption trap. Due to the higher influx, a defined volume of air escapes through the analysis chamber, preventing the system of adventive bacterial or VOC contaminations. In *closed systems*, there is only one pump, which is pushing or pulling the entire headspace volatiles of the bacteria containing chamber on the adsorption material containing volatile-trap. Thereby, in closed *loop* systems air is continuously circulating within the system, while in basic closed *push or pull* systems the air is continuously pulled from the outside. Alternatively to pumps, compressed air can be used. After a certain time interval, the traps are removed from the system. Subsequently, the trapped VOCs are extracted from the trap with solvents (dichloromethane, *n*-pentane, *n*-hexane, methanol, and others), separated in a gas chromatograph (GC), and analyzed using mass spectrometry (MS) (see Sect. 12.3.1). Alternatively, the VOCs can be thermally desorbed from the trap in specific thermal desorption units of a GC and subsequently separated and analyzed using GC/MS.

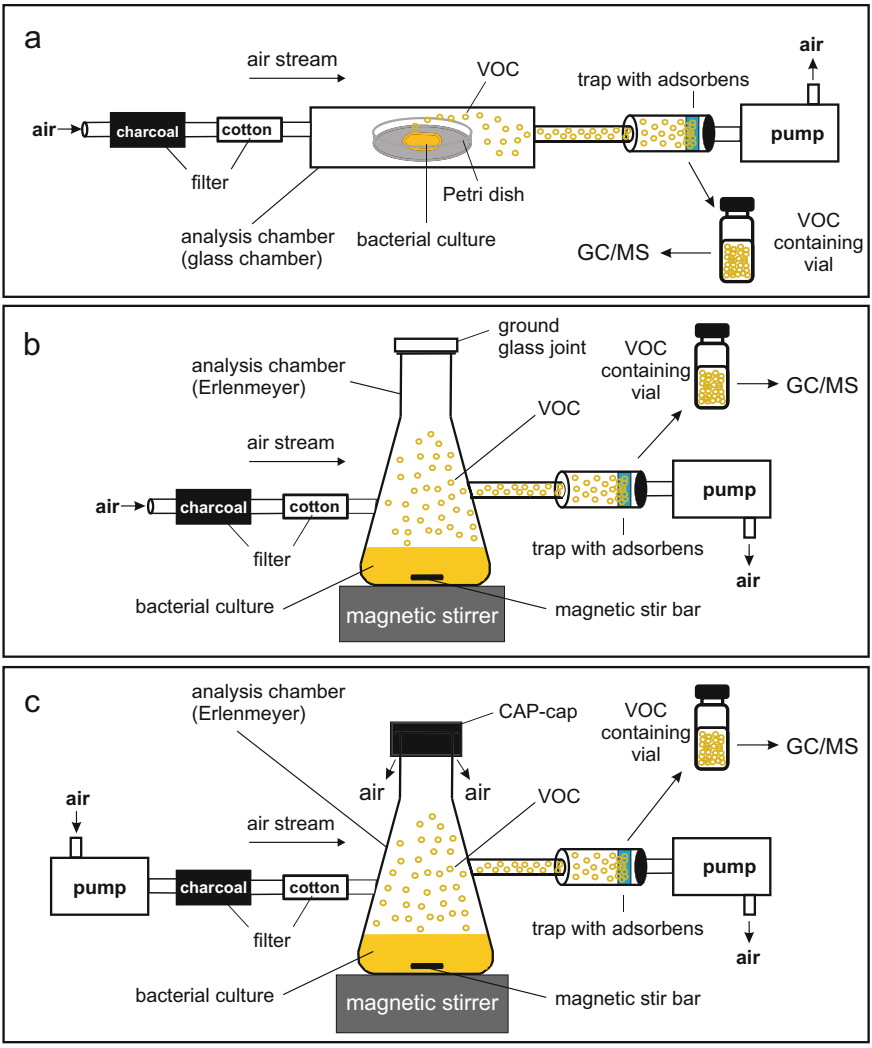

Fig. 12.3 VOC collection in dynamic headspace systems: (**a**) closed VOC collection pull system sampling from a solid bacterial culture, (**b**) closed VOC collection pull system sampling from a liquid VOC culture, and (**c**) open VOC collection system sampling from a liquid culture

12.3 Detection of Bacterial Volatiles

After collecting the volatiles, the next step is to detect the individual bacterial volatiles of the complex volatile mixture. It must be noted that volatiles emitted by bacteria are either of organic (VOCs) or inorganic nature (NH_3, HCN, CO_2, and others) (Piechulla et al. 2017). Most research has focused on the detection of volatile organic compounds; hence, we will only address techniques used for their detection.

Basically, we discriminate between the hyphenated and standardly used GC/MS and alternatively applied direct infusion mass spectrometry techniques.

12.3.1 Standard Detection Technique: Gas Chromatography/ Mass Spectrometry (GC/MS)

The most common and powerful analytical system to detect bacterial VOCs is gas chromatography/mass spectrometry (GC/MS). The main advantage of GC/MS is the separation of VOCs, captured on SPME-fibers or in extracts, within the GC leading to a higher chemical resolution before VOCs are channeled into the MS. Using GC/MS volatiles can be detected in concentrations as low as 0.1 ppt (Blake et al. 2009).

Gas Chromatography (GC) and Multidimensional GC (GC/GC)
In a first step, the individual bacterial VOCs of the complex VOC mixture are separated in the GC. Based on the volatile extraction method used (see Sect. 12.2.1.3, SPME-fiber or traps), there are two approaches to apply the samples on the GC system. While the solvent-extracted VOCs are liquid injected into the GC, the VOCs adsorbed from the SPME fiber are thermally desorbed by direct insertion of the fiber into the injector of the GC. Subsequently, the VOCs are separated on the inner surfaces of a chromatographic column according to their chemical properties. The separation depends on the polarity of these inner surfaces of the column. Most common is the use of DB-5 columns, which are consisting of (5%-phenyl)-methylsiloxane and are recommended for the separation of non-polar VOCs. However, alternatively DB-Wax columns consisting of a polyethylene glycol phase to better separate polar VOCs are also applied.

The application of one column might not sufficiently separate bacterial VOCs of highly complex mixtures. Multidimensional GC (GC/GC), which connects in series two columns with different properties of their inner surfaces, can strongly increase the capacity of the separation (Seeley and Seeley 2013; Bean et al. 2012). In principle, after passing one column the fraction, which contains the VOCs that are not sufficiently separated, is directly transferred onto the second column. To efficiently separate bacterial VOCs, it is recommended that the first column (dimension) has non-polar properties, while the second column (dimension) uses a highly polar phase.

Mass Spectrometry (MS)
After separation in the GC (or GC/GC) the VOCs are transferred through a heated interphase into the mass spectrometer and are subsequently electron ionized (EI). Thereby, a heated filament (cathode) produces electrons, which are accelerated due to a potential of 70 V into the direction of an anode. Due to interaction of the accelerated electrons with the passing VOC, an electron of the VOC is expelled converting the VOC molecule to a positive ion. In addition, the high ionization

energy causes fragmentation of the VOC. The mass spectrometer (usually quadrupole instruments) subsequently selects the resulting ions (molecular ion and fragment ions) of each VOC based on their mass-to-charge ratio (*m/z*). Measurements can be performed using a specific mass range (full scan, e.g., *m/z* 30–250) or by selectively detection of single ions (selective ion monitoring, SIM). SIM is more sensitive compared to the full scan. After passing the quadrupole, the ions are multiplied and detected in an electron multiplier (dynodes or microchannel plate).

To increase the structural information of a bacterial VOC time of flight (TOF) mass detectors are used. TOF instruments measure the mass to charge ratio (*m/z*) of VOCs (of molecular ion and fragment ions) with high mass accuracy by determination their time of flight in a TOF tube. Due to high mass accuracy the prediction of sum formulas of the molecular - and fragment ions are possible, which helps in structure elucidation.

Flame Ionization Detection (FID)
As alternative detector to MS, flame ionization detectors (FID) combined with the GC are used. In FID, the VOCs are ionized in a hydrogen flame. The ion signal is proportional to the carbon content of a VOC. Hence, using FID the retention of the respective VOCs on the column can be evaluated but without further structural information of the VOC. FID detectors are highly sensitive with a wide dynamic range qualifying them for VOC quantification, which is, indeed, more precise compared to mass spectrometry.

12.3.2 Direct Injection Mass Spectrometry Techniques

The most noteworthy shortcomings of GC/MS are the low temporal resolution mainly caused by the chromatographic separation and the pre-concentration processes before analysis. To understand bacterial communication processes within ecosystems, it might be helpful to on-line monitor the emission of bacterial volatiles over a certain time. For this purpose, direct injection mass spectrometry techniques are the preferred experimental setups. Thereby, the VOCs from the headspace of the microbial culture are directly channeled into the MS without using SPME or traps. Besides monitoring dynamics of VOCs, direct mass spectrometric techniques are also applied to search for specific bacterial VOC fingerprints as a diagnostic tool for bacterial identification (Tait et al. 2014; Lough et al. 2017). In the latter investigations, the headspace air is often collected in gas sampling bags and subsequently channeled into the MS. The easy quantification and the low detection limits of direct injection mass spectrometry techniques are advantageous when deciding whether these VOC fingerprint markers are above normal levels as typical in case of diagnosis.

12.3.2.1 Proton Transfer Reaction Mass Spectrometry (PTR-MS)

One direct injection technique to analyze bacterial VOCs is proton transfer reaction mass spectrometry (PTR-MS) (Critchley et al. 2004; Bunge et al. 2008; Kai et al. 2010). PTR-MS allows the simultaneous detection of VOCs corresponding to different chemical classes (N- and S-containing compounds, aldehydes, ketones, alcohols, acids, and ester) with sensitivities from ppm to ppt range. PTR-MS was developed and firstly described by Lindinger and colleagues in 1995 (Hansel et al. 1995; Lindinger et al. 1998). It represents a chemical ionization technique using H_3O^+ primary ions for ionization of the sampled volatiles. H_3O^+ ions are directed into a reaction chamber (drift tube) where protons are transferred from H_3O^+ on the bacterial volatiles. Only volatiles with higher proton affinity than water are ionized. In order to increase the range of detectable volatiles, other molecules than H_3O^+ can be used as primary ions for ionization including NH_3, O_2^+, and NO^+ (Cappellin et al. 2014). Ethylene, a volatile released by *Pseudomonas* species, for instance, is detectable using a PTR-MS instrument modified by a selective reagent ionization (SRI-MS) upgrade that used O_2^+ and NO^+ as primary ions (Cappellin et al. 2014). The product volatile ions (e.g., $M + H^+$ when H_3O^+ is used as primary ion) are subsequently mass analyzed by a quadrupole mass spectrometer. Caused by the soft chemical ionization volatiles are scarcely fragmented and therefore mainly the precursor volatile is detected as *m/z* ratio. Thus, for unequivocal structure elucidation a complementary technique, e.g., GC/MS is needed. When no other technique is available, the identity can be proposed by the use of natural isotopic ratios (e.g., $^{12}C/^{13}C$, $^{32}S/^{34}S$) and literature search. To get profound structural information, PTR-MS can be combined with ion traps and TOF mass analyzers. The use of ion traps (proton transfer reaction ion trap mass spectrometer (PIT-MS)) as mass analyzer has the main advantage to perform fragmentation by collusion induced dissociation in order to identify the volatiles (Steeghs et al. 2007). TOF instruments provide high mass accuracy, which helps to predict sum formulas of the searched volatile. This is especially helpful to identify low molecular weight volatiles, since within this low mass range less possible candidate compounds exist compared to higher mass ranges.

As mentioned above, the great advantage of the PTR-MS technique lies in its high temporal resolution. Thus, it can be used to study the dynamics of the bacterial volatile emission and correlate them to the bacterial growth. These additional information might be very helpful to investigate the biological and ecological significance of bacterial volatiles in nature (Kai et al. 2010; Weise et al. 2012). Furthermore, PTR-MS is used for real-time detection of bacterial VOCs serving as spoilage markers in food sources (Franke and Beauchamp 2017).

12.3.2.2 Selected Ion Flow Tube Mass Spectrometry (SIFT-MS)

Selected ion flow tube mass spectrometry (SIFT-MS) is a technique related to PTR-MS, but instead of higher energy reactions that are applied in PTR-MS, SIFT-MS uses ambient temperature reactions (Cappellin et al. 2014). With detection threshold in the ppb range, SIFT-MS is not as sensitive as PTR-MS. The primary ions (H_3O^+, O_2^+, and NO^+) are delivered via fast-flowing helium into a flow tube in which the bacterial VOCs are introduced to form the product volatile ions (Thorn et al. 2011). These resulting ions are mass analyzed using a quadrupole mass spectrometer. Successful applications of SIFT-MS in the field of bacterial VOCs include VOC profiling for the discrimination of bacterial species including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, *Proteus mirabilis*, *Burkholderia cepacia*, *Streptococcus pyogenes*, and *Enterococcus faecalis* in vitro (Thorn et al. 2011), the quantification of VOCs emitted by in vitro cultures of *S. aureus*, *S. pneumoniae*, and *Haemophilus influenzae* isolated from patients with respiratory diseases (Chippendale et al. 2014), and the identification of *P. aeruginosa* lung infections (Smith et al. 2013).

12.3.2.3 Ion Molecule Reaction Mass Spectrometry (IMR-MS)

The ion molecule reaction mass spectrometry (IMR-MS) combines two integrated MS systems consisting of a conventional EI-MS and a highly sensitive IMR-MS (Dolch et al. 2008). In the first MS unit, krypton, argon, or mercury are electron ionized to form primary ions. In the second MS part, the IMR-MS, these primary ions collide with the neutral VOC molecules. Product ions are formed whenever the ionization potential of the VOC molecules is less compared to the energy potential of the primary ion (Dolch et al. 2008). IMR-MS was successfully applied to differentiate between certain Gram-positive and Gram-negative bacteria according to their VOC profiles (Dolch et al. 2012a, b).

12.3.2.4 Ion Mobility Mass Spectrometry (IMS-MS)

Ion mobility mass spectrometry (IMS) represents a potent and highly sensitive technique to analyze volatile compounds in a range of ppb to ppt (Hill Jr et al. 1990; Baumbach 2006). Bacterial volatiles are directed into the IMS drift tube, a region with a strong, constant electric field that allows the migration of the volatiles. In the drift tube, the volatiles are ionized (radioactive ^{63}N, photo, thermionic, or coronaspray ionization) and subsequently accelerated in the direction of the electric field. Within the drift tube drift gas molecules are located. The accelerated volatile ions collide with these gas molecules that in turn lead to a deceleration of the respective volatile ions. Since the chance is higher that bigger ions compared to smaller ions collide with this gas molecules their velocity in the drift tube is slower.

Thus, the velocity of migration through the electric field depends on the size of molecular mass of the volatiles. IMS can be used as direct injection technique, but is also combined to a GC allowing an increased 2-dimensional separation of volatiles of a complex mixture. However, IMS is not feasible to identify unknown compounds (Baumbach 2006). Applications of IMS in bacterial volatile research are multifarious and comprise the rapid detection of bacteria associated with food sources (Strachan et al. 1995), the characterization of metabolic activity of *E. coli* (Maddula et al. 2009), and the validation of volatile metabolic patterns over different strains of human pathogenic bacteria during growth (Kunze et al. 2013).

12.3.2.5 Secondary Electrospray Ionization Mass Spectrometry (SESI-MS)

In secondary electrospray ionization mass spectrometry (SESI-MS) bacterial volatiles are detected that were ionized by charged particles created by electrospray ionization (ESI) (Wu et al. 2000). Using this gas-phase ionization, bacterial volatiles can be measured in concentrations as small as 1 ppt (Martínez-Lozano et al. 2009). Basically, a solvent is introduced into a needle, which is applied to a high voltage leading to dispersion of the solvent into highly charged droplets. These electrospray droplets charge the bacterial volatiles to form product volatile ions. SESI-MS was reported as most successful ambient ionization method for the analysis of microbial VOCs (Liang et al. 2014), proposed as novel application in real-time clinical diagnosis of bacterial infection (Zhu et al. 2010, 2013a, b; Bregy et al. 2015) and as rapid, accurate detection technology for foodborne pathogens (Zhu and Hill 2013).

12.3.2.6 Atmospheric Pressure Chemical Ionization Mass Spectrometry (APCI-MS)

Another ambient ionization technique to measure bacterial VOCs is atmospheric pressure chemical ionization mass spectrometry (APCI-MS). Avoiding wet chemistry distinguishes APCI from the VOC analysis based on SESI (Liang et al. 2014). Basically, APCI or ambient corona discharge represents a gas phase ionization process (Covey et al. 2009). A high voltage (4–5 kV) applied to a needle creates a corona discharge that forms primary ions from solvent molecules (H_3O^+) and nitrogen (N^{2+}, N^{4+}). The bacterial VOCs are directed to the ionization area at the needle tip and react with the primary ions to form the product volatile ions, which are further transferred to the MS detector. APCI-MS analysis of headspace volatiles was used to differentiate bacterial cultures by MS-fingerprints (Liang et al. 2014) to rapidly detect *Mycobacterium tuberculosis* (Chingin et al. 2016) and the analysis of blood cultures even allowed a rapid, reliable, and cost-effective diagnosis of bacteremia in humans (Chingin et al. 2015). Another advantage of APCI-MS compared to SIFT-MS and PTR-MS is the possibility of fragmentation facilitated identification

(tandem mass spectrometry) of bacterial VOCs. Using ambient corona discharge tandem mass spectrometry, it was, for instance, discovered that the VOC 1-pyrroline might be a marker for *P. aeruginosa* infection (Hu et al. 2016). Beside direct injection APCI-MS can also be combined with GC (Ishimaru et al. 2008). Using a GC/APCI-MS approach VOCs from *E. coli*, *Streptococcus pneumoniae,* and two *S. aureus* isolates were rapidly analyzed after separation (Ishimaru et al. 2008).

12.3.3 Point of Care Devices: Sensors, Biosensors, and Electronic Noses (eNoses)

The development of new and simpler sensors that are portable, sensitive, cheap, and fast has been often regarded as the ultimate goal for this field. Janzen and colleagues developed a colorimetric sensor array to distinguish among a large family of VOCs via employing a low-cost, sensitive colorimetric sensors for the detection and identification of a library of 100 VOCs. The design of an expanded colorimetric sensor array was based on two fundamental aspects: first, each chemically responsive dye must contain a center to interact strongly with analytes; second, each interaction center must be strongly coupled to an intense chromophore (Janzen et al. 2006). To develop such biosensors, Schott and colleagues utilized insect antennae as they are highly sensitive and selective chemical-sensing organs. Schott and colleagues developed an insect antennae-based biosensor to detect VOCs, to be used as a portable device outside the laboratory for field work (Schott et al. 2013). Hurot and colleagues developed a rat odorant binding proteins (OBPs)-based biosensor to detect VOCs in solution by surface plasmon resonance imaging (SPRi). These olfactory biosensors are particularly of interest for trace detection of VOCs present in solution as it is typical in case of body fluids, i.e., saliva, blood, and urine. Such sensing system additionally provides high sensitivity and repeatability (Hurot et al. 2019).

Nowadays, a variety of electronic noses (eNoses) exist on the market. The eNose setup consists of an array of sensors simulating the human olfactory response (Staples 1999). eNoses were proved to be a useful non-invasive technique capable of detecting VOCs. These devices allow imprinting of an odor on its sensor array. When the sensors are exposed to a gas, the polymer absorbs the gas and swells, during which the distance between the conductive carbon particles increases, thus increases the resistance of the sensor material. Such change in resistance is transmitted to a computer with the pattern of change in the sensor array being used to detect the gas. eNoses are powerful at distinguishing between non-identical samples and provide rapid results with on-site sampling. However, they are limited due to temperature and humidity sensitivity. Furthermore, they can only detect VOCs that are present in their reference database, and are therefore limited to screen known volatiles without the chance of identifying unknown compounds (Wilson and Baietto 2009). Newer eNoses such as zNose was developed to detect VOCs with

higher sensitivity and speed, based on ultra-fast GC and surface acoustic wave (SAW) sensor (GC-SAW). The zNose technique also provides on-line measurements without any sample pretreatment (Staples 2000; Devkota et al. 2017; Oh 2018). These devices have great potential to prove the presence or absence of specific VOC markers with limited sample preparation.

12.4 Identification of Bacterial Volatiles

The detection of VOCs is followed by their identification. A reliable and unambiguous identification of VOCs is often very challenging, since bacterial species can produce rich and diverse VOC profiles, which can even include novel and unknown compounds (von Reuss et al. 2010; Lemfack et al. 2016; Vidal et al. 2017). This section discusses issues related to the identification of bacterial VOCs mainly detected in GC/MS analysis.

The electron ionization (EI) in the mass spectrometer is usually performed with 70 V resulting in highly reproducible mass spectra, which represent fragmentation patterns that are characteristic for each compound. The fragmentation patterns of the compounds of interest can be compared with databases (e.g., National Institute of Standards and Technology (NIST), Wiley). These databases give suggestions in a sequence of putative compounds starting with the highest score based on similarities of the mass spectrum of interest compared to mass spectra compiled in the database. However, compounds of the same family (e.g., structural isomers or members of a homologue series) can show very similar EI mass spectra. Thus, the similarity search of compounds using databases can result in wrong identification, e.g., shown for the identification of n-dodecane, which appeared only at the seventh position in similarity search using NIST database with 94% similarity (Fig. 12.4). Calculation of retention indices, for instance the Kováts index, helps to reduce the selection of potential candidates (Kováts 1958). The calculated Kováts indices can be compared to indices of compounds published in databases (NIST, Pherobase, the Adams library). In this respect, iMatch represents a further development as retention index

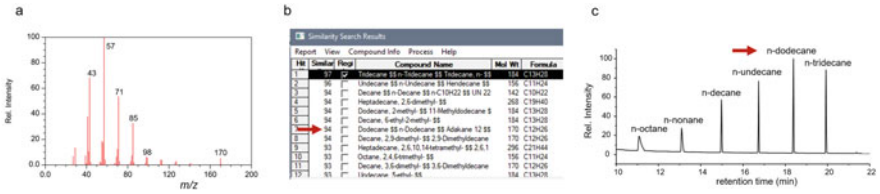

Fig. 12.4 Identification of n-dodecane. Mass spectrum of *n*-dodecane (**a**) revealed that dodecane was only at the seventh position in similarity search using NIST Library with 94% similarity (**b**). In order to distinguish between the single alkanes, e.g., the first hit tridecane with 97% similarity, co-elution experiments with authentic standards confirmed the identification of *n*-dodecane (**c**)

tool for analysis of GC/MS data and to confirm identification of VOCs among false hits (Zhang et al. 2011).

Finally to unequivocally identify compounds of interest, it is essentially needed to verify database propositions by performing co-elution experiments with commercially available authentic standards. Novel and unknown VOCs can be enriched in dynamic HS systems (Sect. 12.2.1.3) to be subsequently identified using nuclear magnetic resonance spectroscopy and chemical synthesis. The identity of the unknown VOC has to be confirmed with comparisons of its mass-spectral and retention properties with those of the newly synthesized compound. Unfortunately, this state-of-the-art methodology was not always carefully conducted in the past, which may have led to falsely identified compounds.

12.5 Multivariate Data Analysis of Bacterial VOCs

Data analysis always depends on the biological question. In order to evaluate the data, univariate statistical tests depending on *P* values and multivariate methods are applied. Univariate statistics have shown their limitation over the years (Malley et al. 2013). Thereby, the question on the validity of considering $P \leq 0.05$ as a threshold for statistical significance in research is still debated (Benjamin et al. 2018; Gagnier and Morgenstern 2017). Correction to multiple testing has been introduced to control the number of false positive due to the multiple hypotheses testing that researches use when working with VOC-omic data (Benjamini and Hochberg 1995; Broadhurst and Kell 2006). However, multivariate analysis has been proven to be useful in analyzing and interpreting bacterial VOC-omic data (Thorn et al. 2011; Lemfack et al. 2016). To assign differences in VOC production among bacterial strains in an untargeted routine, multivariate data analysis could be performed on VOC quantification data. Furthermore, the differential VOC profiles from different microbes combined with the analysis of differential levels of gene transcription might be useful for probing biosynthetic pathways leading to volatiles production.

Multivariate analysis can be conducted using two different approaches (unsupervised and supervised). For the sake of data exploration and to get a first impression of the data, unsupervised methods in which you do not label, classify, or categorize the data points are usually used. One of the most favored unsupervised methods is principal component analysis (PCA). A PCA basically provides a way to visualize complex data while reducing its multidimensionality. Using PCA derived from GC/MS data, the skin-borne bacterial families *Staphylococcaceae* and *Corynebacteriaceae* were clearly distinguishable by their VOC profiles (Lemfack et al. 2016). To identify variables represented by VOCs that contribute to clustering patterns, the loading plots can be further examined from the corresponding score plot (Farag et al. 2018). Another method to investigate the clustering of the samples is hierarchical clustering analysis (HCA), which is based on an algorithm that groups similar samples into clusters, thus it may mirror PCA clustering (Lubes and Goodarzi 2017). Processing the mass spectra of Gram-positive bacteria by

hierarchical clustering facilitated, for example, differentiation between certain bacterial species (Dolch et al. 2012b; Lemfack et al. 2016). Other famous clustering algorithms are k-means clustering, which is a centroid-based clustering method used to partitioning samples into a defined number of clusters (Hageman et al. 2006) and k-medoids (partitioning around medoids, PAM) clustering, which is similar to k-means but additionally uses data points as centers (Kaufman and Rousseeuw 1990).

Compared to unsupervised modeling, supervised multivariate analyses attempt to discover the relationship between an input and a target (Maimon and Rokach 2005), i.e., volatiles abundance and certain response or effect. They were proven to be advantageous due to its ability to establish a classification model in which VOCs are tested as predictors for their correlation with a certain response or class. Multivariate techniques such as linear discriminate analysis (LDA) and partial least-square discriminate analysis (PLSDA) are used to achieve that purpose. LDA is a fast and powerful technique that is used to find a linear function based on the VOCs to be able to differentiate between different groups (Vera et al. 2011). However, it can be only applied when samples number is bigger than the number of VOCs. PLSDA is found to be superior, if working with highly collinear data, for instance, in order to find marker VOCs that are important in distinguishing between different groups (Wold et al. 2001). Importantly, PLSDA requires model optimization and validation parameters be assessed, i.e., ANOVA, prediction power and permutation tests to avoid erroneous results and data overfitting (Westerhuis et al. 2008; Brereton and Lloyd 2014; Farag et al. 2018).

Furthermore, researchers need to recognize that not all VOCs will necessarily follow linear pattern. Therefore, non-linear methods accompanied by machine learning have been developed over the years. Commonly used non-linear methods are the support vector machine (SVM), kernel-PLSDA, and artificial neural network (ANNs) (Mahadevan et al. 2008; Amato et al. 2013). These tools are powerful predictors for VOCs providing classification of samples and became, for instance, popular in diagnostics of diseases. However, model validation and quality control are critical to assure the robustness of the model and its prediction level (Xia et al. 2013).

In summary, different analytical methods are used in VOC analysis, which should be accompanied by suitable methods for data analysis. To achieve this, several factors should be carefully considered (e.g., method sensitivity, sample complexity, range of VOCs measured using the chosen method, and use of uni- or multivariate analyses depending on the number of samples and the goal of the study). In that regard, application of a receiver operating characteristic (ROC) analysis for those volatiles that show the strongest differences among specimens can help to verify their validity as markers for strain discrimination. ROC analysis is directly and naturally related to cost efficient analysis of diagnostic decisions and is routinely applied for biomarker discovery in diseases pathogenesis (disease vs. non disease) and can be further considered for VOC analysis in bacteria (Ammar et al. 2017). Integration of different kinds of data (metadata) pertinent to the samples and VOCs across different instruments are essential to be implemented.

12.6 Conclusion

Over the last 15 years, researchers recognized the significance of bacterial VOCs as infochemicals in microbial ecology and restarted to investigate their production and emission. Using various in vitro headspace VOC collection systems bacteria showed a great capacity to emit a broad range of VOCs. Thereby, the bacterial species analyzed to date represent only the "tip of the iceberg" compared to the number of bacterial species existing on Earth. During VOC analysis, bacteria were mainly mono-cultivated under nutrient rich conditions neglecting important ecological parameter such as nutrient conditions in the habitat and biotic interaction. The major tool used for detection and quantification of bacterial VOCs is GC/MS due to their high chemical resolution. However, in order to increase the temporal resolution and in turn to monitor the dynamics of VOC emission during bacterial growth, a series of various direct injection mass spectrometry techniques are available. The identification of VOCs is very challenging since mass spectral databases only give suggestions based on similarity. Unequivocal identification always includes the comparison with authentic standards. For data analysis and interpretation of bacterial VOCs, different algorithms in multivariate analysis ought to be employed to help in understanding the biological significance of bacterial VOCs in nature and to expedite the discovery of a VOC as marker for certain bacterial species.

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Chapter 13
Using Bacteria-Derived Volatile Organic Compounds (VOCs) for Industrial Processes

Peter Kusstatscher, Tomislav Cernava, and Gabriele Berg

Abstract Microbial volatiles harbour an extensive spectrum of chemical compounds. Bacteria-derived volatile organic compounds (VOCs) were studied for decades in order to uncover their role in microbial interactions and to decipher their importance in the communication with other organisms. In the frame of extensive screening approaches, various VOCs with growth inhibiting effects against pathogenic bacteria and fungi were found in the recent years. These discoveries not only have a great importance for the general scientific knowledge, but also open the way for many technological applications of those molecules. The application of the discovered bacterial volatiles in industrial decontamination processes provides new alternatives for conventional chemicals. Moreover, they might facilitate the reduction of harmful, toxic and cancerogenic chemicals and widen the toolbox for a broader spectrum of biological decontamination agents. In addition, VOCs have a great potential for microbiome management and control, and can be applied as bio-preservatives, -pesticides, and fumigants.

Keywords Volatile organic compounds · Decontamination · Microbiome management · Bio-fumigation · Bio-preservation

13.1 Introduction

Microbial communities live in close relation with each other and can colonize higher organisms. Many hosts were shown to harbour well-defined and often highly diverse communities, which include bacteria, fungi as well as archaea. Such host-associated microbiota fulfil important functions such as growth promotion or specific defence mechanisms against pathogens (Berg 2009; Mendes and Raaijmakers 2015). Our knowledge related to the interactions of bacteria within the community and their

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hosts gradually improved during the last years. Studies have shown that prevalent defence mechanisms rely on competition for nutrients, antibiosis through soluble molecules, and microbial volatile organic compounds (VOCs) (Berg 2009; Raaijmakers et al. 2009; Mulero-Aparicio et al. 2019). Especially VOCs were found to be mediators of various interactions between microorganisms such as bacteria and fungi but also across kingdoms, e.g., between microorganisms and plants (Effmert et al. 2012; Kanchiswamy et al. 2015). Microbial VOCs typically occur as complex mixtures of relatively low-weight lipophilic compounds and are collectively often described as the "volatilome" (Maffei et al. 2011; Kanchiswamy et al. 2015). The potential of reaching greater distances within the soil or surrounding environment makes them the ideal candidates for signal transduction (Maffei et al. 2011). The functions of volatiles are numerous, e.g., they influence physiological processes (e.g. nitrification), they support metabolic reaction (e.g. as electron acceptors or donors), they are quorum sensing/quenching molecules, they serve as food source within the food chain when integrated in organic matter, or they provide other so far unknown functions (Effmert et al. 2012).

A broad spectrum of different volatile molecules can be released by microorganisms, small inorganic and organic compounds (<120 Da) or more complex VOCs (120–300 Da). While smaller molecules were studied for a long time, larger compounds received more attention just in the last years (Effmert et al. 2012). More than 800 different volatiles were identified so far and it was shown that volatile spectra of single microbial strains can be rather simple (<10 VOCs) or extremely complex (>50 VOCs) (Kai et al. 2007, 2010; Effmert et al. 2012). By applying modern technologies such as solid phase microextraction (SPME), gas chromatography, in combination with mass spectrometry (GC/MS) or other trapping methods such as proton transfer reaction, secondary electron spray ionization, an increasing number of volatiles can be detected (Wenke et al. 2012). These detection methods can be coupled with efficient screening assays in order to target volatiles with specific characteristics, e.g., antimicrobial effects against defined phytopathogens (Cernava et al. 2015).

The most dominant classes of molecules emitted by fungi are alcohols, ketones, hydrocarbons, terpenes, alkanes, and alkenes (Chiron 2005). In contrast, bacteria prominently release alcohols, alkanes, alkenes, ketones but also esters, pyrazines, sulphides, and lactones (Wenke et al. 2012). Terpenoids, short-chain alcohols, and acids can especially be found in the volatilome of various *Streptomyces* species (Citron et al. 2012), *Pseudomonas* species emit particularly C9-C16 alkanes and alkenes (Effmert et al. 2012).

The effects of these highly diverse molecules as well as detailed modes of action are largely unknown although their potential for various applications has been discovered a long time ago. Already over 60 years ago Dobbs and Hinson (1953) described the fungistatic effect of bacterial volatiles on soil-borne fungi (Dobbs and Hinson 1953). In addition, Strobel and colleagues showed the great applicability of fungal VOCs from *Muscodor albus* and brought it for mycofumigation applications into the market (Strobel 2006). Volatiles produced by *Streptomyces griseus* were shown to induce an early sclerotium formation in *Rhizoctonia solani* and *Sclerotium*

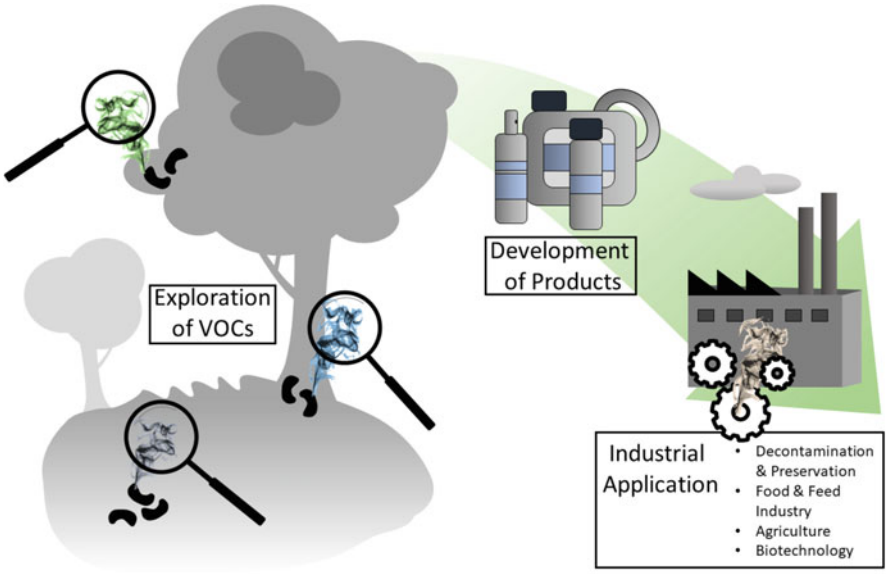

Fig. 13.1 Schematic representation of the exploration of natural VOCs from environmental samples for product development in industrial processes. Natural environments often harbour highly diverse microbial communities that include members with useful traits. The identification of beneficial microorganisms and their secondary metabolites, including VOCs, is a crucial step for the development of improved products for various industrial applications

cepivorum as well as reduce sporulation in *Gloeosporium aridum* (McCain 1966). Soon single molecules such as but-3-en-2-one from *Streptomyces griseoruber* were found to have a strong growth inhibiting effect on fungi (Herrington et al. 1987). Additionally, acetamide, benzaldehyde, benzothiazole, 1-butanamine, 1-decene, methanamine, and phenylacetaldehyde produced by several different genera of bacteria were found to have fungistatic effects (Zou et al. 2007). The discovery of these molecules is not only interesting from the scientific point of view, but also provides the basis for novel, untapped industrial applications. However, the development of competitive products requires a targeted exploration of natural resources (Fig. 13.1). Specific examples for the high potential of microbial volatiles to replace conventionally used disinfectants in the industry will be presented in this chapter. In addition, various applications based on VOCs for the food, animal feed, or other biotechnological sectors that were developed in the recent years will be shown. This brief overview is meant to serve as an inspiration for further developments that will be based on these highly promising molecules.

13.2 Potential of VOCs for Industrial Applications

13.2.1 Food Production Industry

Nowadays the food industry faces serious problems with providing sufficient food products for the growing market. Moreover, the products should meet not only the quality demands of the consumer but also the safety standards of the legislation. Therefore, food research groups all around the world investigate new techniques to develop environmentally friendly and safe methods for reducing food contaminations. Consumer protection legislations restrict the usage of harmful chemicals used in the past, but also provide the basis to introduce novel, improved methods into the market. Apart from advanced physical treatments such as cold plasma treatments, UV-light treatments, or high-voltage pulsed electric field treatments (Niemira 2012; Boziaris 2014), bacterial volatiles provide a broad spectrum of promising decontamination agents with high efficiency and low environmental impact.

Recently, alkyl-substituted pyrazines, VOCs found in the spectrum of, e.g., *Paenibacillus polymyxa*, were identified as mediators of antimicrobial effects, reducing the growth of plant and human pathogens (Cernava 2012; Rybakova et al. 2016). The low mammalian toxicity and the activity against a broad spectrum of pathogens at low concentrations are the two major factors making pyrazines, especially 2,5-bis(1-methylethyl)-pyrazine, and 5-isobuthyl-2,3-dimethylpyrazine potential bio-based fumigants (Janssens et al. 2019).

For meat products, in particular fresh meat, efficient preservation is key to guarantee a long shelf life. While physical parameters such as temperature, atmospheric oxygen, moisture, or light can be controlled, microbial colonization can still be a challenging factor (Zhou et al. 2010). Techniques like refrigeration, chemical preservation, active packaging, and high hydrostatic pressure are state-of-the-art methods in the industry (Zhou et al. 2010; Dave and Ghaly 2011). However, natural bio-preservatives, providing the requirements for green label products which attract more customers, are interesting for industrial applications. Schöck et al. (2018) investigated the applicability of 5-isobuthyl-2,3-dimethylpyrazine and 2-isobutyl-3-methylpyrazine in a maltodextrin carrier on processed meat. Following the application of an optimized formulation, the microbial pressure was decreased up to 95% and therefore the shelf life increased substantially (Schöck et al. 2018).

In a similar approach, the antimicrobial effect of 5-isobutyl-2,3-dimethylpyrazine utilizing its fumigation potential on hatching eggs was evaluated (Kusstatscher et al. 2017). Industrialized production of fertilized eggs requires a high level of sterility to provide a good chicken development. Therefore, egg shells are disinfected using fumigated formaldehyde (Williams 1970). Formaldehyde was linked to harmful health effects and therefore handling is challenging (Wartew 1983). In the recent study, the researchers were able to achieve comparable decontamination rates of egg shells using fumigated pyrazine compounds. Successful decontamination was also confirmed using micrographic observations (Kusstatscher et al. 2017).

In addition to the aforementioned applications, postharvest fruit treatment harbours an increasing market of sustainable treatments replacing harmful chemicals. Fruit availability all around the year requires a prolonged storability after harvest, which was so far mostly achievable with either high energy consumption or the application of various preservatives. However, consumers' demands for low residues restrict the industry in potential chemical use (Sivakumar and Bautista-Baños 2014). Therefore, a wide range of natural compounds including microbial volatiles are continuously explored for their application potential. Volatiles are especially applicable, because their application reduces required volumes and artificial atmosphere packaging can provide protection along the marketing chain (Mari et al. 2016). In a targeted approach, different compounds produced by two *Bacillus* strains were shown to reduce *Penicillium crustosum* on citrus fruit to an extent comparable to modified atmospheric packaging (Arrebola et al. 2010). Additionally, volatiles of *Streptomyces platensis* F-1 reduced disease incidence of gray mold in strawberries by 73% (Wan et al. 2008) and volatiles of *Streptomyces globisporus* JK-1 were explored to control *Botrytis cinerea* in tomato (Li et al. 2012a).

13.2.2 Animal Feed Treatments

Microbial contaminations of animal feed are not only an economic problem, but also an important threat for animal health. Once such contaminants enter the food chain, they can also become serious risk factors for humans. The main sources of animal feed can be contaminated by fungi (*Penicillium, Aspergillus, Fusarium,* and *Alternaria*) as well as bacteria (*Campylobacter* and *Salmonella*) and often require the destruction of large quantities of soy, corn, or wheat (Kabak et al. 2006; Malorny et al. 2008; Rodrigues and Naehrer 2012). Researchers found that 30 up to 100% of the 475 million tons of animal feed consumed in the European Union each year are affected by microbial contaminations to some extent (Rodrigues and Naehrer 2012; Pinotti et al. 2016). While fungal contaminants primarily produce various mycotoxins, *Salmonella* pathovars can cause large disease outbreaks in animal farming. One aggravating factor is the extended persistence of these microorganisms in different substrates, including animal feed (Jones and Richardson 2004; Magossi et al. 2019). Moreover, a total of 380 deaths and 19,000 hospitalizations are caused by *Salmonella* infections each year in the USA and additional cost of $2.3 billion is related to *Salmonella* for the food and feed industry (CDC 2011). Animal feed, apart from slaughter houses, were identified as one major sources of infections and therefore surveillance programs have been set up around the globe (Li et al. 2012b; Magossi et al. 2019).

Currently, animal feed that is potentially contaminated with *Salmonella* is treated with formaldehyde or various organic acids (Carrique-Mas et al. 2007; Jones 2011). While formaldehyde is toxic and an environmental hazard, organic acids employed for the decontamination are mostly safe alternatives, but substantially decrease the palatability of animal feed (Wales et al. 2010). Microbial volatiles that naturally

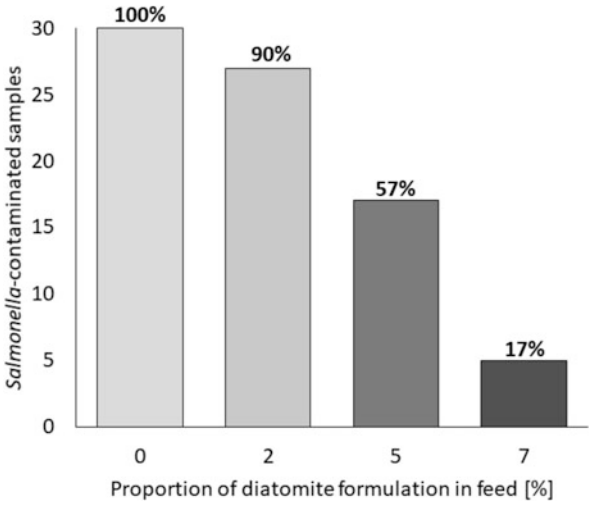

Fig. 13.2 Removal of *Salmonella* in contaminated soy with bacterial VOCs. Diatomite enriched with 5-isobutyl-2,3-dimethylpyrazine (30% w/w content) was used to treat 30 contaminated samples with three different concentrations, respectively. Viable *Salmonella* was recovered after 7 days of incubation at representative storage conditions. Contaminated proportions are indicated above the columns

deactivate the relevant pathogens are a promising replacement for current treatments. In a recent study conducted by the authors, it was shown that the application of distinct alkylpyrazine compounds that are often produced by *Bacillus* and *Paenibacillus* species can be used to remove contaminating *Salmonella* from animal feed (Fig. 13.2). Diatomite was employed for the first time to deliver the antimicrobial 5-isobutyl-2,3-dimethylpyrazine to contaminated soy feed. The number of contaminated samples was substantially reduced when higher proportions of the alkylpyrazine-enriched diatomite were added. This example provides evidence for the applicability of VOCs for animal feed treatments. Further research is however required to optimize the dosage and application of the active ingredients as well as to perform a full toxicologic assessment of the compounds.

13.2.3 Decontamination in Industrial Production Facilities

Many industrial sectors face problems related to bacterial and fungal contaminations at their production facilities. Such contaminations affect industrial sectors beyond food and animal feed production. Especially bioreactors are prone to contaminations because they provide suitable environments not only for the cultivated microorganisms, but also for undesired contaminants. In this context, large-scale photobioreactors represent a production environment with aggravating process conditions. Under the current cultivation conditions for microalgae unwanted, co-occurring microorganisms such as bacteria, fungi, zooplankton, or other weedy microalgae potentially disturb mass cultivations and can lead to a complete collapse of the cultures and thus rise production costs (Benemann and Oswald 1996; Kim et al. 2014). Currently, the most common decontamination procedures include

rinsing of the reactors with sodium hypochlorite or the application of hydrogen peroxide. However, it was found that the low stability and the high reactivity of the disinfectant are often disadvantageous for various process environments. Novel, efficient alternatives could improve industrial-scale microalgae cultivations by providing more reliable methods for large-scale photobioreactor decontaminations. In a recent study, the applicability of VOCs was assessed for this purpose (Krug et al. 2019). Alkylpyrazines that were also found to be also applicable for other agricultural and industrial processes (Kusstatscher et al. 2017; Schöck et al. 2018; Mülner et al. 2019) were assessed in terms for their employability in photobioreactor decontaminations between subsequent fermentation batches. It was show that the application of 5-isobutyl-2,3-dimethylpyrazine in microalgae cultures was highly efficient and led to significant reductions of cell viability of common eukaryotic contaminants. This preliminary study provides the basis to further explore the applicability of highly efficient VOCs for decontaminations of industrial process environments. They could provide and unlock environmentally friendly alternative for broad scope of applications including algae production.

13.3 The Potential of VOCs for Future Developments

Due to the manifold exploitable characteristics of VOCs, their implementation in industrial processes will likely gain momentum and find more applications in various production processes. Several promising examples of VOCs applicability were provided in this chapter. It can be expected that many more applications will likely be developed in the future. For example, VOCs can be used to manage desired functional properties of food products regarding safety and preservation issues, organoleptic or health properties (Cocolin and Ercolini 2015). Especially postharvest applications provide a near-natural environment to deploy VOCs-based treatments. Their application can reach efficiencies that are in the range of conventional treatments that are currently employed (Mari et al. 2016). Microbial volatiles have a great potential for medicinal applications or the treatment of medical facilities. The increasing occurrence of antibiotic-resistant microorganisms is globally a serious threat for mankind (Andersson 2003; Knapp et al. 2009). For several decades, it seemed that the battle against many infectious diseases was won. This was disproved when various bacteria-caused diseases reappeared as a leading cause of death worldwide. Recently, the World Health Organization (WHO) released an updated list of threatening microorganisms, which have become multi-resistant towards available antibiotics (Tacconelli et al. 2017). Despite the frequent bacteriostatic effect of many microbial volatiles, distinct substances have also been found to be involved in the induction of antibiotic resistances (Avalos et al. 2018). By interfering with cellular mechanisms, indole was found to activate an ampicillin efflux pump in *Pseudomonas putida* and ammonia (NH_3) was shown to increase the membrane durability against tetracycline in *Escherichia coli* (Bernier et al. 2011; Molina-Santiago et al. 2014). Therefore, the impact of volatiles in cellular processes needs

to be considered in further research. However, it was generally found that important drivers for the development of antibiotic resistance are the misuse and overuse of antibiotics in humans and animals (Conly 1998; Ventola 2015; Moore 2019). Furthermore, current decontamination methods facilitate the emergence of resistant microorganisms and can thus aggravate the situation in hospitals (Vandini et al. 2014). Therefore, the transfer of natural regulation processes to critical, man-made environments like hospitals could be a viable strategy to improve the current situation. In this context, the employment of natural mixtures of microbial volatiles might provide a vital strategy to counteract resistance formation. The application of mixtures instead of single molecules was previously shown to prevent developing resistances in agriculture (Elderfield et al. 2018).

First studies have shown that volatiles of plant-associated bacteria harbour the potential to counteract important human pathogenic bacteria including important health care-associated infections, e.g., caused by *Stenotrophomonas maltophilia* (Cernava 2012). However, the employment of such substances would require novel methods of delivery, especially in environments that are inhabited by humans. The design of efficient formulations should consider to the composition of natural volatilomes that are found in healthy environments, e.g., the phyllosphere of plants. This could facilitate a prolonged efficacy of novel, nature-based decontamination methods.

Apart from decontamination applications, VOCs also harbour potential for novel applications in agriculture. Due to their importance in interspecies communication and defence strategies of microorganisms, studies suggest their potential for microbiome management and control in the rhizosphere of plants. Targeted application of VOCs could promote the formation of a desired microbial community (Kanchiswamy et al. 2015; Fierer 2017; Liu and Brettell 2019). Additionally, VOCs produced by plant endophytes were found to enhance plant resistance to pathogens and protect against herbivores. Therefore, novel biocontrol strategies are possible (Strobel 2006; Fierer 2017). Furthermore, the study of pathogen associated VOCs could be utilized as an early detection tool for plant associated diseases and has also great potential for medical applications (Sankaran et al. 2010; Bos et al. 2013).

13.4 Conclusion

Microbial VOCs provide a broad spectrum of novel, bioactive molecules and are therefore an important source of new decontamination strategies for industrial applications. The exploration of VOCs is not only important to increase our understanding of microbial interplay, but also provides the potential to transfer identified mechanisms of this powerful molecules to new application fields. In the past years, detailed studies of microbial interaction compounds led to the discovery of newly, so far unnoticed, antimicrobial molecules. This is not only a leap for the scientific community but has also an impact on possible technological implementations of those molecules. During the last decade, several new processes relying on the

intensive research put into VOCs discovery were developed. They are often considered as environmentally friendly and safe alternatives to traditional chemical compounds. However, they undergo the same registration processes to guarantee their safety before application. Nevertheless, the trend towards natural compounds provides a basic rationale to further explore the applicability of microbial volatiles in industrial processes.

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Chapter 14
Formulation and Agricultural Application of Bacterial Volatile Compounds

Rouhallah Sharifi and Choong-Min Ryu

Abstract Bacterial volatile organic compounds (VOCs) play critical roles in biological communications in the agroecosystem. At an optimum concentration and application duration, VOCs improve plant growth and health, and are therefore considered as a new source of agrochemicals. To overcome the limitation of chemicals in nature, encapsulation of VOCs increases their stability and enables their slow-release. However, choosing the best encapsulation method and optimizing the encapsulation process are critical for obtaining a suitable formulation. Several encapsulation methods such as emulsion extrusion, coacervation, spray-drying, and freeze-drying have been exploited for preparing the formulations of volatiles such as pheromones, essential oils, and plant and microbial volatiles. Carbohydrates are the main wall materials for encapsulation, but proteins and synthetic polymers have many advantages and are therefore used as substitutes for carbohydrates or are mixed with carbohydrates in large-scale capsule production. Virus particles are also considered as bio-molecular carriers for the efficient delivery of agrochemicals, especially in the soil. It is necessary to control various quality indices of BVC capsules such as particle shape and size, encapsulation efficiency, release pattern, and chemical interaction between VOCs and the wall material. Altogether, encapsulation is a promising technology for the efficient application of VOCs in the field.

Keywords Agrochemicals · Microencapsulation · Slow-release · Volatile organic compounds

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14.1 Introduction

Agriculture business forms a large part of the world trade and is directly responsible for food production and feeding the increasing world population (Palocci et al. 2017). However, a significant part of the attainable agricultural yield is lost because of pests, diseases, and weeds. These invading agents threaten food safety and security all around the world. Plant protection experts and farmers exploit several methods, including pesticide application, to manage plant pests. Approximately 4.6 million tons of chemical pesticides are applied annually across the world (Zhao et al. 2017). Nonetheless, approximately 35% of the yield, amounting to approximately 550 billion dollars, is lost each year because of biotic stresses (Agrios 2005). Because this yield loss is much higher in the absence of chemical pesticides, the latter represent an indispensable part of the agricultural industry. However, the application of pesticides is very inefficient. Approximately 90% of the applied pesticides do not reach their targets and are lost because of drift, evaporation, surface run-off, hydrolysis, chemical transformation, and microbial degradation (Nuruzzaman et al. 2016; Zhao et al. 2017). Pesticides applied directly to the soil are mainly absorbed by the soil organic matter and never reach the root developmental zone (Lamberth et al. 2013). Therefore, to reach the target organism and obtain satisfactory harvest results, a large amount of pesticides is applied to the host plant, which is much higher than its minimum effective concentration (MEC) on target organism but lower than its minimum toxic concentration (MTC) on plant. However, application of a large volume and dosage of pesticides increases the risk of creating pesticide resistant races of pests and pathogens.

Researchers and R&D units of agrochemical companies use two approaches to minimize the effects of pesticides on the environment and human health. The first approach is to develop new pesticides that are highly effective against the target pests but have minimal negative impact on non-target organisms. Natural products, especially ecologically important communication signals, such as plant and microbial volatiles, insect pheromones, and antibiotics, are considered as an important source of safe and environmentally friendly agrochemicals. In the second approach, researchers try to develop formulations that are more efficient in transferring pesticides to the target organisms. New formulations, such as attract-and-kill and sustained release formulations, increase the bioactivity and bioavailability of volatile compounds (Sharifi and Ryu 2018a). When used in combination, these two approaches facilitate the development of natural pesticides with greater application efficiency by transferring these compounds to the target organism at an appropriate concentration and over a sufficient period of time. New pesticide formulations such as Karate Zeon® and Demand CS®, containing the active ingredient (a.i.) lambda-cyhalothrin, and Seltime®, containing the a.i. Pyraclostrobin, are commercial outputs of these efforts (Huang et al. 2018; Wege et al. 1999).

Ecologically important metabolites, such as VOCs, a source of new agrochemicals, are highly sensitive to chemical interaction, degradation, and evaporation, and take time to reach their optimum influence (Chinchilla et al. 2019; Xie et al. 2009).

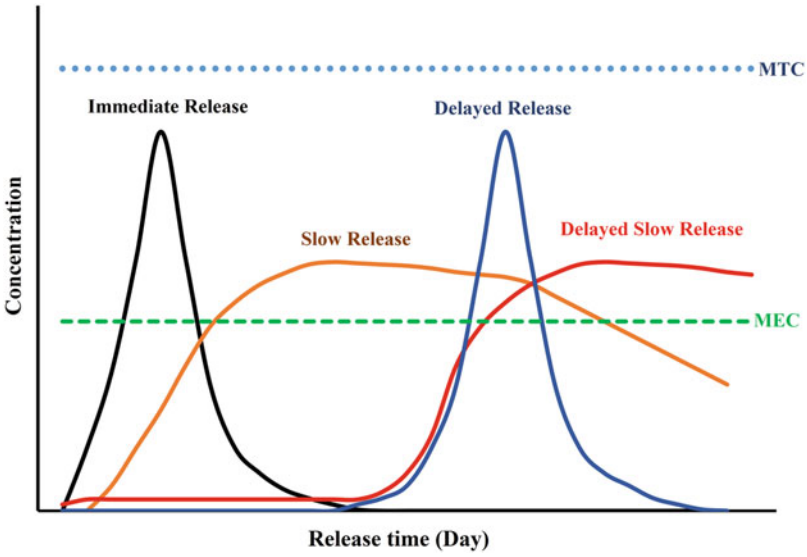

Fig. 14.1 Release patterns after field application of agrochemicals. Conventional pesticide formulations release the active ingredient (a.i.) immediately after application. The amount of a.i. released is much higher than its minimum effective concentration (MEC) but lower than its minimum toxic concentration (MTC). The delayed-release formulation releases the a.i. at once after exposure to the environmental stimulus, whereas the slow-release formulation releases the a.i. in a steady state for a long time at a concentration approaching MEC. The delayed-slow-release formulation has features of both the delayed-release and slow-release formulations

Controlled-release formulations are suitable for the delivery of VOCs. Two types of controlled-release technologies are known: delayed-release and slow-release (Fig. 14.1). In the slow-release formulation, sensitive core materials are encapsulated within a semipermeable polymer shell known as a wall or crust. These permeable capsules not only preserve core materials but also theoretically support their sustained release over a long period of time at an optimum concentration to achieve high bioactivity. The amount of core material inside the capsule could be higher than the MTC, but the encapsulation process can be optimized to release it at the MEC over a sufficient time period. Notably, some core materials such as VOCs and hormones may negatively impact plant growth beyond their MEC. Therefore, their release should be optimized and checked meticulously. In the delayed-release formulation, core materials are encapsulated in an environmentally sensitive polymer shell to induce immediate release by various environmental stimuli such as sunlight, temperature, pH, and shear stress (Trachsel et al. 2016; Ullah et al. 2015). Delayed-release formulations provide the opportunity to release core materials at a specific time or place. Moreover, these two technologies can be combined to form delayed-slow-release formulations. In these formulations, core materials are double-sandwiched inside layers of permeable and environmentally sensitive shells. The delayed-slow-release formulation releases its a.i. in a specific environment over a

long period of time. Yu et al. (2019) modified nanocapsules by adding tannic acid to increase their foliage adhesion. This modification increased their retention rate on cucumber leaves by more than 50% (Yu et al. 2019). Furthermore, it is possible to encapsulate volatile emitter organisms, which exhibit a sustained release of a volatile blend (Parafati et al. 2017); in this case, the encapsulation process needs to be optimized for microbe survival.

Encapsulation is an emerging technology in several research and industrial fields, such as food, flavour and fragrance, and drug delivery (Cuma 2018; Jafari et al. 2008). Several methods have been developed and optimized to produce controlled-release capsules in different sizes and application types. Encapsulation technology is gaining interest in the agricultural sector, especially in the field of plant protection, and has been exploited to develop new formulations of chemical pesticides and insect pheromones (Nuruzzaman et al. 2016; Prasad et al. 2017; Wang et al. 2018; Zhao et al. 2017). Here, we review the potential and examples of controlled-release technology in the field application of VOCs. These volatile compounds are considered as gaseous fertilizers or pesticides for plants and have a great impact on plant growth and health (Chinchilla et al. 2019; Choi et al. 2014; Sharifi and Ryu 2018b; Song and Ryu 2013).

Capsules have different sizes and structures, based on the raw materials and production process. Capsules have a single core if the encapsulation process is slow, but are multicore if the encapsulation is rapid. Some encapsulation processes produce a sphere, which has an internal matrix instead of a core. While capsules release the a.i. in lag phase, spheres show a more steady-state release. Capsules are also divided into different categories, depending on their size. Capsules with a diameter greater than 1000 μm are known as macro-capsules and are suitable for application to soil using a seeder. Capsules ranging in size from 1 to 1000 μm are considered as microcapsules, and those <1 μm or <100 nm in at least one dimension are known as nanocapsules (Duhan et al. 2017; Zhao et al. 2017). However, these capsules, especially nanocapsules, have different definitions, as size is not the only defining characteristic of nanomaterials. Nanomaterials are ultra-fine materials with a high surface area-to-volume ratio, which confers new and specific features (Pascoli et al. 2018; Zhao et al. 2017). The size of capsules affects their application method, loading capacity, and slow-release activity (Jafari et al. 2008; Pascoli et al. 2018).

The following steps are involved in the process of encapsulation: (1) selection of the encapsulation technology; (2) selection of the best polymer as wall material, based on the encapsulation technology and core material properties; (3) mixing the a.i., polymer, emulsifier, and supplementary additives; (4) emulsification; (5) encapsulation; and (6) quality control. In the following sections, we discuss the advantages and disadvantages of common wall materials and encapsulation technologies, with examples of the encapsulation of VOCs and similar core materials.

14.2 Encapsulation Materials

Several materials are commonly used for encapsulation including carbohydrates, gums, proteins, and synthetic polymers. Out of encapsulation materials, wall materials must be cost-effective, biodegradable, and biocompatible, with a low viscosity in high solid content, good emulsification, and high core material retention potential. Ideal wall materials also protect the a.i. from degradation, provide a uniform particle size, and support the sustained release of the core material. Here, we introduce the main wall materials.

14.2.1 Carbohydrates

Carbohydrates are cheap and highly bioavailable polymers. Well-known carbohydrates used for encapsulation include starch, cellulose, and chitosan. Chitosan exhibits greater advantages than other carbohydrates for use in plant protection, as it can induce systemic resistance to plant pathogens (Desaki et al. 2018; Sharp 2013). Chitosan forms capsules and entraps the a.i. in the polyanionic solution of triphosphates, or covalently crosslinks with glutaraldehyde. Chitosan nanocapsules containing the nitric oxide donor, *S*-nitroso-mercaptosuccinic acid, induced markedly greater systemic tolerance to salt stress in maize plant than the un-encapsulated a.i. (Oliveira et al. 2016). These nanocapsules showed an encapsulation efficiency (EE) of 97% and a mean capsule size of 38 nm (Oliveira et al. 2016). Chitosan has also been exploited for the encapsulation of the plant hormone gibberellic acid, with a mean particle size of 134 nm and an EE of 90% (Santo Pereira et al. 2017). This formulation increased seed germination, leaf surface area, and root volume of tomato plants under field conditions.

14.2.2 Modified Carbohydrates

Carbohydrates exhibit good core material retention but low emulsification. It is possible to improve the emulsification property of carbohydrates by modifying their structure. For example, starch is a cost-effective bioavailable polymer, but it shows low solubility in cold water and low emulsification property. However, starch derivatives, produced by the hydrolysis and chemical or enzymatic modification of starch, exhibit better encapsulation potential than starch. Maltodextrin is derived from the partial hydrolysis of starch. Although, maltodextrin exhibits low emulsification and core material retention activity, it protects the a.i. efficiently. Therefore, maltodextrin is commonly used as a secondary carrier in combination with other wall materials such as Arabic gum (AG), which exhibits high emulsification and core material retention potential (Alves et al. 2014; Botrel et al. 2015; Kang et al. 2019).

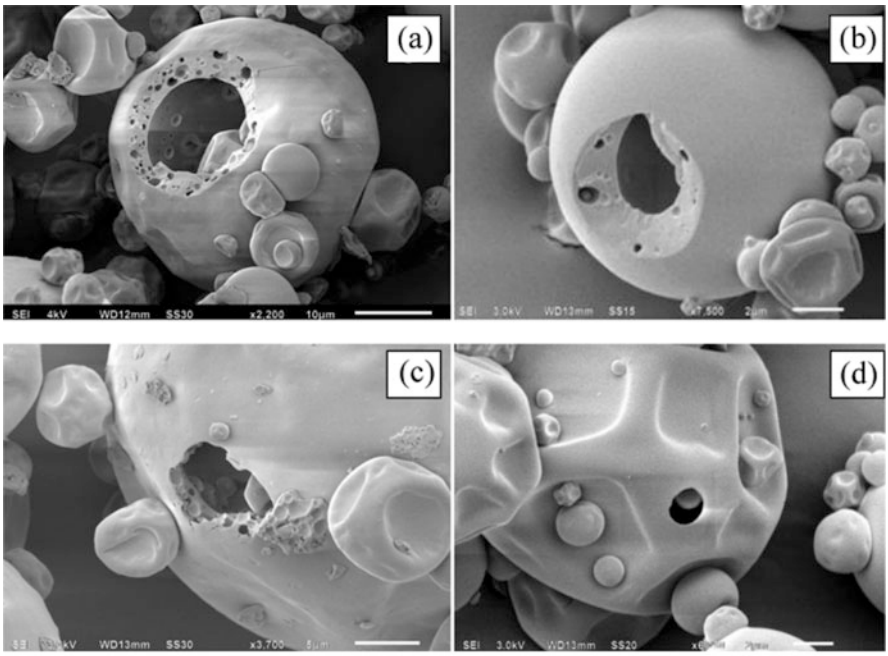

Fig. 14.2 Altering the wall material and encapsulation process affects microcapsule structure and encapsulation efficiency (EE). (**a**) Capsules containing 1 part essential oil (EO), 2 parts Arabic gum (AG), and 3.6 parts maltodextrin (M) (w/w) have a thick but slightly deformed wall. (**b**) Capsules containing 1 part EO, 3 parts AG, and 3.6 parts M show a thick and smooth wall. (**c**, **d**) Capsules lacking M (**c**) or AG (**d**) result in a thin wall and deformed shape. Formulation (**b**) showed the highest EE and stability during storage (Alves et al. 2014)

Combination of maltodextrin and AG leads to the production of smooth and spherical capsules (Fig. 14.2) (Alves et al. 2014). Moreover, maltodextrin exhibits low hygroscopy and decreases the water activity (a_w) of the prepared capsules. Low a_w reduces capsule coalescence and microbial degradation. The presence of maltodextrin in β-caryophyllene formulations decreases the a_w of microcapsules and increases the protection of the a.i. (Alves et al. 2014). Dextrose equivalent (DE) is an important index of the efficiency of maltodextrin. DE lower than 20 units produces better retention. Increasing DE value decreases the permeability of capsules to oxygen, which protects reactive molecules such as VOCs from oxidation, whereas high DE values increase the cracking of capsules, resulting in the loss of core materials (Turchiuli et al. 2005).

Cyclodextrin is a circular oligosaccharide derived from the enzymatic modification of starch. It contains seven glycopyranose moieties, which form a trapezoid cone with an internal diameter of 5–8 Å (Filippa et al. 2008; Neri-Badang and Chakraborty 2019; Sanchez et al. 2011). The interior of the trapezoid cone is lipophilic, whereas the exterior is hydrophilic; consequently, cyclodextrin is a suitable candidate for the formulation of lipophilic VOCs. Cyclodextrin also has a

long storage stability and has been successfully used for the encapsulation of methyl salicylate (Me-SA) as an anti-inflammatory agent (Filippa et al. 2008). Me-SA is a well-known BVC for its effect on plant health and growth (Park et al. 2007; Wang et al. 2019a).

Starch can be chemically modified by the addition of succinic acid or octenyl as a side chain, which converts it to an amphiphilic compound (Botrel et al. 2015; Jafari et al. 2008). Chemically modified starch (CMS) has an appropriate emulsification property and can retain high amounts of lipophilic VOCs. The viscosity of CMS is greater than that of AG (Shahidi and Han 1993). Therefore, CMS is a good candidate for spray-dried encapsulation and has been used for the slow-release of D-limonene for 28 days (Márquez-Gómez et al. 2018).

14.2.3 Gums

Gums such as alginate, AG, xanthan, gellan, and carrageenan are very commonly used polysaccharides in different encapsulation technologies (Jafari et al. 2008; Taheri and Jafari 2019).

AG exhibits high water solubility (up to 50% w/v) and emulsification (Cui et al. 2013). Given its low viscosity and high solid content, AG is a suitable wall material for microencapsulation, especially in the spray-drying technology. These properties are a result of its unique structure. AG constitutes saccharides, such as rhamnose, arabinose, and galactose, and contains 2% protein (Sanchez et al. 2018; Turchiuli et al. 2005). However, the availability of natural AG is limited, which increases its price over time (Sanchez et al. 2018). Nowadays, AG is combined with other wall materials, such as polysaccharides in encapsulation technology, especially in the spray-drying method (Alves et al. 2014).

Alginate is a cheap, non-toxic, and biocompatible heteropolysaccharide extracted from brown algae and bacteria (Cuma 2018; Taheri and Jafari 2019), and it is commonly used as a wall material in the emulsion extrusion method. Alginate is a polymer of 1,4 linked β-D-mannuronate (M) and α-L-guluronate (G) residues, with high solubility (782 g/L) in water (Cuma 2018; Li et al. 2016). Alginate is present in the form of the negatively charged COO^- ions in solution (Heuskin et al. 2012). Calcium ions (Ca^{2+}) form crosslinks with the carboxylate groups of the G residue in neighbouring polymer chains to form a three-dimensional (3D) gel matrix via a process known as ionic gelation (Heuskin et al. 2012). Thus, the relative proportion of G to M is an important factor affecting the quality of alginate as a wall material. It is possible to use different calcium sources such as calcium lactate ($C_6H_{10}CaO_6$), calcium gluconate ($C_{12}H_{22}CaO_{14}$), and calcium chloride ($CaCl_2$) for encapsulation, depending on their availability, price, and gelation efficiency. However, calcium chloride results in a higher EE (99%), smaller capsule size, and longer release than other calcium sources (Cuma 2018). Cations such as barium (Ba^{2+}), copper (Cu^{2+}), lead (Pb^{2+}), and cadmium (Cd^{2+}) work better than Ca^{2+}, but they are not commonly used because of their toxicity (Cuma 2018; Heuskin et al. 2012). Barium chloride

($BaCl_2$) has been used to prepare alginate capsules of Thiram® fungicide (Singh et al. 2009).

Alginate encapsulation is not suitable for some environments containing Ca^{2+} chelates or gel degrading agents. Therefore, it is not compatible with most of the commercial fertilizers that contain chelating agents. To solve this problem, alginate beads are coated with cationic polymers such as poly L-lysine, gelatine, and chitosan, which bind to free COO^- anions and form a secondary shell layer on alginate beads (Natrajan et al. 2015; Valladares et al. 2016). Coated alginate beads have a denser and firmer crust but are larger in size (Li et al. 2016). Some studies suggest the use of secondary carbohydrates, such as starch, chitosan, and cellulose, for improving the slow-release property of alginate beads (Natrajan et al. 2015; Neri-Badang and Chakraborty 2019; Nörnberg et al. 2019). For example, compared with alginate as the sole wall polymer, the use of cellulose in addition to alginate increased the release period from 11 to 43 days, and EE from 72% to 85%. Ionic gelation using alginate has been successfully exploited for the encapsulation of Me-SA as an herbivore-induced plant volatile. This formulation supported the sustained release of BVC for 15 days. Field evaluation showed that encapsulated Me-SA controlled the wheat aphid *Sitobion avenae* either directly or by attracting its predator, Syrphid fly (Wang et al. 2019a). Additionally, the plant auxin phenylacetic acid (Li et al. 2016) and semiochemicals such as E-β-farnesene, E-β-caryophyllene (Heuskin et al. 2012), and sulcatol (Valladares et al. 2016) have been encapsulated in alginate beads.

14.2.4 Proteins

Proteins such as gelatine, whey protein concentrate, whey protein isolate, and casein are frequently used as wall materials (Eghbal and Choudhary 2018; Xiao et al. 2014). Proteins are water-soluble and have a high emulsification ability. These properties depend on the isoelectric point of proteins. Gelatine is commonly used as positively charged polymer in the coacervation encapsulation method (Eghbal and Choudhary 2018). Gelatine is an ampholyte containing approximately 13% positively charged amino acids and 12% negatively charged amino acids (Lee et al. 2014). However, the net charge of gelatine depends on the pH of the solution. It has a negative charge below its isoelectric point. Gelatine contains amino acids such as glycine, proline, and hydroxyproline, which induce systemic tolerance to abiotic stresses (Chen et al. 2011; Chun et al. 2018). Therefore, gelatine is preferred over other polymers for the encapsulation of tolerance-inducing VOCs.

14.2.5 Synthetic Polymers

Synthetic polymers such as polylactic acid (PLA), polyglycolides (PGA), and poly (lactide-co-glycolides) (PLGA) are used in industrial encapsulation (Pascoli et al. 2018; Shen et al. 2018; Wang et al. 2018; Yu et al. 2019). The physiochemical and slow-release properties of capsules can be controlled in synthetic polymers. Some of these polymers have been approved by the Food and Drug Administration (FDA) and can be used in the food and agriculture market, especially for post-harvest pest management (Valletta et al. 2014). These polymers are commonly used in capsule formulations of pesticides. PLGA nanocapsules are 30–50 nm in diameter and are absorbed by plant roots and fungal mycelia via cell membrane endocytosis (Palocci et al. 2017; Prasad et al. 2017; Valletta et al. 2014). Therefore, it is possible to apply PLGA nanocapsules as systemic slow-release pesticides. Encapsulation of *Spodoptera litura* pheromone in monomethoxy poly(ethylene glycol)–poly(-ε-caprolactone) (MPEG-PCL) copolymers produces capsules (374 nm diameter), which release pheromone for up to 14 days (Chen et al. 2018).

14.2.6 Plant Viruses

Viruses are biological nanostructures of various shapes and biochemistry. Nucleic acids of viruses are naturally encapsulated in the internal cavity of rod, filamentous, and icosahedral virus particles. These cavities, especially rod and icosahedral cavities, can be exploited to encapsulate and deliver sensitive agrochemicals such as VOCs in the field (Fig. 14.3). Virus nanoparticles (VPNs) have been used for the encapsulation of nematicides, pesticides, and herbicides (Cao et al. 2015; Chariou et al. 2019; Chariou and Steinmetz 2017; Guenther et al. 2018). At least one VPN, Solvinix®, which contains a herbicide has been registered by BioProdex (Chariou and Steinmetz 2017). Solvinix is derived from the *Tobacco mild green mosaic virus* (TMGMV). Solvinix works better than PLGA and mesoporous silica nanoparticles in the delivery of the nematicide to the root developmental zone. Non-capsulated nematicides are adsorbed by the organic matter in the topsoil, whereas VPN reaches a soil depth of 30 cm (Chariou et al. 2019). To produce VPNs, virus particles are multiplied on their natural host and extracted by standard methods. Pores in virus particles can be induced by decreasing Ca^{2+} and magnesium (Mg^{2+}) concentrations using ion chelators such as ethylenediaminetetraacetic acid (EDTA). These porous particles uptake agrochemicals, according to their molecular weight and charge. Increasing Ca^{2+} concentration closes the pores to form encapsulated VPNs. Red clover necrotic mosaic virus has been used for the encapsulation of Abamectin®, which penetrates deep into the soil and controls nematodes better than the un-capsulated nematicide (Cao et al. 2015).

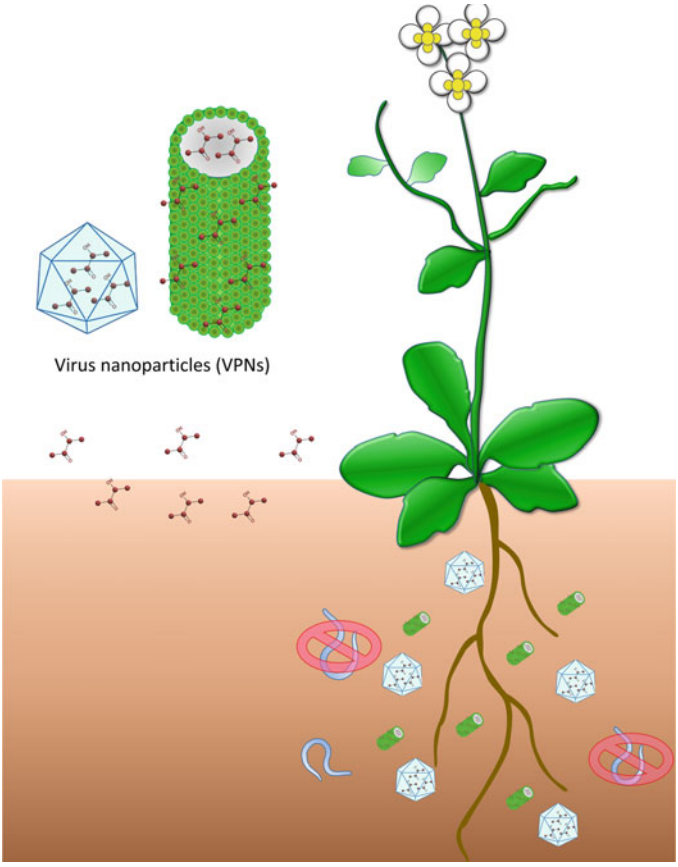

Fig. 14.3 Virus nanoparticles (VPNs) can adsorb, encapsulate or conjugate agrochemicals, and deliver them to root developmental areas. VPN can be selected based on their shape, cavity volume, and interacting amino acid residues. These particles prevent bioactive ingredients to be adsorbed by organic matter, delivering them to the rhizosphere. Encapsulated compounds such as bacterial volatile organic compounds will be protected from evaporation

14.3 Encapsulation Process Technology

Emulsification plays an important role in downstream processes and determines the shape, size, and permeability of the capsule and sustained release of capsule products. EE is dependent on the emulsification capacity of wall materials and the use of emulsifier and oil. Among wall materials, proteins and protein-containing gums such as AG have suitable emulsification capacity, which depends on the pH of the emulsion (Sanchez et al. 2018; Taheri and Jafari 2019). However, polysaccharides have low EE. Therefore, it is important to improve the EE of polysaccharides by modifying their chemical structure, adding emulsifiers, or combining them with proteinaceous carriers. Emulsifiers such as sodium dodecyl sulphate (SDS),

polyvinyl alcohol (PVA), Span 80, and Tween 20 have been used for improving the emulsification of wall materials (Kong et al. 2009; Liu et al. 2016; Wang et al. 2018). Moreover, hydrophilic core materials such as some VOCs require a plant or mineral oil and an emulsifier to produce a homogenous emulsion. Emulsification occurs by mechanical stirring, homogenization, or sonication (Jafari et al. 2007). Ultrasonic devices have a greater acceptance for emulsification than other devices. Premix membrane emulsification is an innovative technology used for the production of uniform particles. In this technology, the emulsion is passed through a membrane to prepare nano- or microparticles, based on the type of membrane and the pressure and viscosity of emulsion. Membrane emulsification works without any intense ultrasonic or heat treatment and therefore is suitable for sensitive core materials (Liu et al. 2016).

14.3.1 Spray-Drying

Spray-drying has a high potential for low-cost mass-production of microcapsules on an industrial scale (Botrel et al. 2015). In spray-drying, an emulsion of core and wall materials is passed through a peristaltic pump and sprayed using an atomizer in a heat chamber with heat gradient for a few seconds (Alves et al. 2014; Botrel et al. 2015; Jafari et al. 2008). Heat dries the capsule surface rapidly (Jafari et al. 2008), and dried capsules are then collected from the bottom of the chamber. This method requires a good emulsion with a high solid content and low viscosity to facilitate easy spraying through the nozzle; otherwise, droplets do not shrink properly, resulting in high permeability. Therefore, AG, CMS, and proteins are suitable candidates for microencapsulation using the spray-drying method (Alves et al. 2014; Márquez-Gómez et al. 2018; Shahidi and Han 1993).

There are two atomization apparatuses, a nozzle atomizer and a rotary disc atomizer (Botrel et al. 2015). In the nozzle atomizer, the size of particles depends on the emulsion viscosity, pump pressure, and nozzle pore diameter. Although the nozzle produces uniform capsules, it is not suitable for highly viscous emulsions (Botrel et al. 2015). By contrast, the rotary atomizer can atomize viscous emulsions; however, the size of capsules depends on the viscosity of the emulsion and disc rotation speed (Huang et al. 2004).

Temperature regulation is a necessary step in spray-drying, as it affects the particle size and core material retention and stability. However, reports show that a brief exposure to temperatures as high as 200 °C does not affect sensitive core materials such as volatile compounds or living organisms (Botrel et al. 2015; de Barros Fernandes et al. 2014; Huang et al. 2017). Nevertheless, temperature optimization is critical for obtaining the highest capsule quality. The temperature of the heating chamber can be managed by optimizing the inlet and outlet temperatures. Inlet temperature generally ranges from 150 to 200 °C (Botrel et al. 2015). Low temperature has a less negative effect on core materials than high temperature, but it does not allow proper drying of capsules. The resulting capsules are deformed and

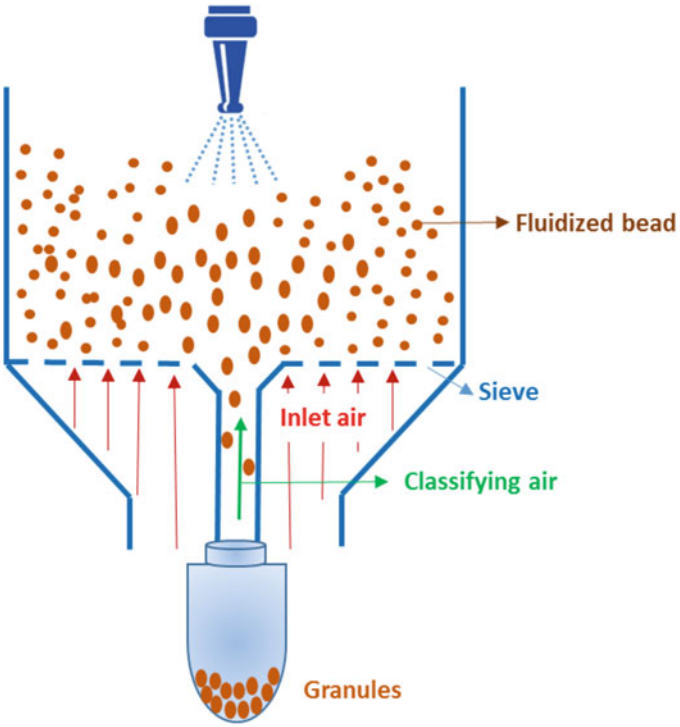

Fig. 14.4 Fluidized bed method exploited to cover microcapsules with extra wall materials. Microcapsules can be suspended by warm air flow and atomizer nozzle covered them by layer of sticky and wettable materials. These procedures microcapsule agglomeration preparing them to be collected by classifying air through a collecting funnel

have a high a_w and reduced storage durability. Freeze-drying is used as an alternative to spray-drying for highly sensitive core materials. However, freeze-drying has low EE and is not suitable for the economical scale-up of microcapsule production (Quispe-Condori et al. 2011).

The powder produced by the spray-drying method can be subjected to further modification, especially if it is not wettable. Microcapsules are covered by a layer of hydrophilic materials using a fluidized bed method. In this method, capsules are placed on a sieve at the bottom of the heat chamber, a flow of hot or warm air suspend particles in a chamber (Fig. 14.4). Air velocity should be higher than the gravitational force to suspend particles in the air. Simultaneously, a nozzle at the top of the chamber sprays a solution of sticky compounds over the particles. This layer of liquid on capsules dries by the hot air flow. Consequently, capsules aggregate to form larger granules. Agglomeration of microcapsules using the fluidized bed technology does not change the encapsulation indices noticeably but increases the water solubility of capsules (Botrel et al. 2015; Turchiuli et al. 2005).

14.3.2 Extrusion

There are two methods of extrusion: heat melt extrusion and ionic gelation. The heat melt extrusion method makes pellets containing the a.i. This method is not suitable for heat-sensitive VOCs. However, it is possible to add these compounds towards the end of the process to reduce the time of exposure to high temperature (DiNunzio et al. 2010). By contrast, ionic gelation is performed at room temperature and is therefore suitable for BVC encapsulation. In this method, an emulsion of core and wall materials is dropped into an ionic solution to form jelly capsules with a uniformly large size. Factors such as needle pore diameter, viscosity, and distance between the needle and solution affect the capsule size (Heuskin et al. 2012; Nörnberg et al. 2019). To reduce the capsule size, emulsion is sprayed onto the solution using a spraying apparatus such as the Nisco VAR13 encapsulator (Pahwa et al. 2015). Electrostatic dispersion or vibration is exploited to produce highly uniform capsules (Heinzen et al. 2004). Furthermore, utilization of emulsifiers such as PVA and dioctyl sodium sulphosuccinate, followed by homogenization, can lead to smaller and multicore capsules with higher EE (Kumar et al. 2014; Neri-Badang and Chakraborty 2019; Wang et al. 2018). Extrusion can be performed either by external or internal gelation methods. External gelation is more commonly used than internal gelation. In external gelation, an emulsion of alginate and core materials is dropped into $CaCl_2$ solution. Then, Ca^{2+} ions penetrate into the alginate beads and induce gelation from the exterior (Cuma 2018; Li et al. 2016; Pahwa et al. 2015). Factors such as the ratio of Ca^{2+} to COO^- and the time of gelation can be optimized. In internal gelation, an emulsion of alginate, core materials, and $CaCl_2$ is homogenized and dropped into an organic solvent (Silva et al. 2006). Because of the reduction in pH, $CaCl_2$ is ionized and bound by the COO^- group of alginate. In both methods, beads can be hardened using a covalent crosslinker, such as formaldehyde, and then washed and air-dried.

14.3.3 Porous Membrane

Porous membranes with a specific pore diameter can be used to produce uniform micro- or nanocapsules. Shirasu porous glass (SPG) is available in different pore sizes ranging from 0.1 to 20 μm. The emulsion of core and wall materials is passed through the SPG, with a specific pore diameter, using a pump pressure. SPG with a pore size of 0.1 or 0.3 μm has been used to produce Azoxystrobin®-containing PLA nanocapsules, with a mean diameter of 130 and 353 nm, respectively (Yao et al. 2018). Uniform spherical PLGA nanocapsules of Fluazinam® have been produced using SPG; these capsules were 300–600 nm in diameter and released the pesticide for 288 h (Wang et al. 2019b). Porous membrane is an innovative technology for large-scale production of uniform nanocapsules. This encapsulation technology does not require heat and therefore is suitable for the encapsulation of VOCs.

14.3.4 Coacervation

Coacervation is considered as the oldest encapsulation process and uses liquid–liquid phase separation. Simple coacervation produces capsules, based on the salting-out phenomenon. In this method, water is extracted from the polymer solution using salt or alcohol. The final particle is hardened by formaldehyde or glutaraldehyde. In complex coacervation, two oppositely charged polymers, usually proteins and polysaccharides, encapsulate the core material by electrostatic interaction. Gelatine–AG is the most well-studied polymer combination for complex coacervation. However, gelatine can be substituted by other animal or plant proteins such as whey protein, casein, and silk fibroin, whereas AG can be substituted by chitosan, alginate, carrageenan, agar, and carboxymethyl cellulose. Several variables such as pH, polymer concentration, polymer ratio, and temperature have a significant effect on the quality of coacervate capsules (Eghbal and Choudhary 2018; Xiao et al. 2014). Optimized complex coacervation of dodecyl acetate, an insect pheromone, achieved using a combination of whey protein and AG at pH 3.5 with a wall-to-core mass ratio of 3:1, released the pheromone for 30 days (Yu et al. 2012). The same wall materials were used for the complex coacervation of dodecanol, an insect pheromone. Glutaraldehyde works better than formaldehyde and supports constant release of the pheromone (Kong et al. 2009).

14.4 Quality Control of Encapsulation

The quality of microcapsules is affected by several factors related to raw materials and the encapsulation process. These factors can be optimized to achieve an efficient capsule formulation. To optimize the process of encapsulation, we need indices related to the quality of the produced capsules such as EE and loading efficiency, size and shape determination methods, analytical methods such as gas chromatography followed by mass spectrometry (GC-MS) and proton transfer reaction MS (PTR-MS, to determine the release of the a.i. over time), and methods such as Fourier transform infrared spectroscopy (FTIR, to determine the chemical interaction between core and wall materials) (Kumar et al. 2014; Natrajan et al. 2015). Light or scanning electron microscopy and particle size analysers are used to determine the size and shape of capsules (Kumar et al. 2014; Wang et al. 2019b). Sometimes, the addition of colour can help visualize the capsules under a light microscope. However, some colours may have a negative effect on the encapsulation process. The EE and loading capacity of capsules represent the efficiency of polymerization and encapsulation to load a suitable amount of the core material into the capsule (Kumar et al. 2014; Nörnberg et al. 2019). Maintaining the slow-release property and increasing the EE and loading capacity help to reduce the cost of capsule production and downstream application processes. EE and loading capacity can be calculated using Eqs. (14.1) and (14.2), respectively, as shown below:

$$\text{EE} = \frac{\text{Total active ingredient} - \text{uncapsulated active ingredient}}{\text{Total active ingredient}} \times 100 \quad (14.1)$$

$$\text{Loading} = \frac{\text{Total active ingredient} - \text{uncapsulated active ingredient}}{\text{Total weight of encapsulated product}} \times 100 \quad (14.2)$$

To estimate the un-capsulated a.i., a known amount of the formulated capsule should be suspended in a suitable solvent and centrifuged. The amount of a.i. in the supernatant represents the un-capsulated a.i., and it can be measured by spectrophotometry or chromatography. In the emulsion extrusion method, the amount of a.i. remaining in $CaCl_2$ represents the un-capsulated core material. Most of the encapsulated formulations absorb water after application, and the speed and capacity of water absorption can be calculated using Eq. (14.3) (Cuma 2018; Li et al. 2016; Nörnberg et al. 2019):

$$\text{Swelling} = \left(\frac{\text{Weight after water absorbtion}}{\text{Weight of dry formulation}} - 1\right) \times 100 \quad (14.3)$$

Two methods are used to determine the release of VOCs over time. In this first method, the amount of volatiles released by capsules into the headspace (HS) is measured using volatile collection techniques such as the HS–solid phase microextraction (SPME), stir bar sorptive extraction (SBSE), and Porapak Q. The extracted VOCs are loaded into the analytical equipment and measured by GC-MS and PTR-MS (Sharifi and Ryu 2018a; Valladares et al. 2016; Wang et al. 2019a). In the second method, the released core materials are collected from the dialysis bag submerged to the proper solvent (Nörnberg et al. 2019; Wang et al. 2018). These core materials are then detected and quantified using a spectrophotometer or by GC-MS or high performance liquid chromatography (HPLC), based on the chemistry of VOCs (Li et al. 2016).

14.5 Conclusion

VOCs generally function as signalling molecules in intra- and interspecific communication (Bailly et al. 2014; Piechulla et al. 2017; Sharifi and Ryu 2018b). These metabolites are either consumed as a source of nutrition, growth regulator, or act as toxins to suppress the growth of organisms (Bailly et al. 2014; Kai et al. 2009; Meldau et al. 2013; Sharifi and Ryu 2018b). Additionally, VOCs are potentially a new source of agrochemicals (Sharifi and Ryu 2018a). Pure VOCs show promising results in improving plant growth and suppressing pests and diseases in the field (Choi et al. 2014; Song and Ryu 2013; Wang et al. 2019a). However, VOCs have some limitations and need to meet specific prerequisites before being introduced into

the market. VOCs are highly sensitive to oxidation, evaporation, and chemical interaction (Sharifi and Ryu 2018a). Therefore, it is important that formulations protect VOCs from these adverse phenomena. Furthermore, VOCs take time to induce a physiological change in the receiver organism (Xie et al. 2009), but pure VOCs evaporate easily in the open field. An ideal formulation should also emit VOCs for several hours or days to reach the optimum effect. Encapsulation and sol–gel are promising technologies for the protection and sustained release of VOCs in the open field (Sharifi and Ryu 2018a; Wang et al. 2019a). Here, we explained the raw materials and methodology for encapsulating VOCs. However, only a few examples of BVC encapsulation are known (Parafati et al. 2017; Wang et al. 2019a), and we used examples of similarly sensitive chemicals, such as pheromones and plant essential oils, to elucidate the weaknesses and strengths of encapsulation technologies. It is possible to optimize the methodology and results of the BVC encapsulation process based on other sensitive volatile chemicals. However, specific core and wall materials need specific optimization.

Application of encapsulated VOCs is an important issue. Previously, we explained BVC application methods such as chemigation, application using a conventional seeder or sprayer, and post-harvest application (Sharifi and Ryu 2018a). The size and wettability of capsules play an important role in choosing an appropriate application method. The products of emulsion extrusion are sufficiently large to be applied using a conventional seeder or centrifugal fertilizer spreader. Nevertheless, most of the encapsulation methods produce micro- or nanocapsules that are suitable for spray application on crop rows. Studies show the systemic movement of nanocapsules in plant tissues (Palocci et al. 2017; Prasad et al. 2017; Valletta et al. 2014). Notably, VOCs have a systemic effect per se and activate systemic signals that not only translocate throughout the plant but also transfer to neighbouring plants (Kalske et al. 2019; Sharifi et al. 2018). Therefore, full coverage of plant tissues or even spraying on all rows may be not necessary for VOCs. Empirical data are needed to confirm these hypotheses. Currently, we are in the initial phase of transferring these high potential metabolites from the laboratory to the open field condition. Any gaps in information need to be filled using more empirical data.

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