Signaling and Communication in Plants

James D. Blande
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Deciphering Chemical Language of Plant Communication

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Signaling and Communication in Plants

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Preface

The concept that plants interact with other organisms by emitting volatile chemicals is well established. Through the emission of volatile chemicals, plants advertise their physiological condition, which can provide valuable information to organisms that detect the odorous bouquet. Take, for example, volatiles emitted by herbivore-damaged plants; they can be received by neighbouring plants that increase their defences, foraging herbivores that can opt to forage either in the same area or elsewhere depending on perceived competition, predatory insects for which the chemicals indicate the presence of their prey, and numerous other members of the community. Volatile chemicals, as well as being emitted by plants, can be detected by plants and elicit various responses. Therefore, plants are not only communicators delivering a volatile presentation to an audience, but are members of an audience receiving chemically encoded information from other sources.

In the last decades, efforts to understand and decipher the chemical language of plants have increased substantially. In this book, we traverse three parts that deliver cutting-edge knowledge on several critical components of volatile-mediated plant communication. Part I covers the production and emission of volatile chemicals and the complexity of chemical messages that plants deliver. Attention is given to the temporal dynamics of plant volatile emissions, the role of abiotic factors in regulating emissions and the impact of multiple stresses as interacting inducers of emissions. A picture begins to build about the complexity of the volatile bouquets emitted by plants and how they can be viewed as an informative chemical language. Throughout the book, there is a focus on chemical ecology, which comes to the fore in Part II. In Part II, a clear picture is developed of the myriad interactions mediated by plant volatiles, spanning interactions between plants and herbivores, predatory and parasitic insects, hyperparasitoids, vertebrates, other plants, pollinating insects, microorganisms and mutualists. Interactions occurring both above- and below-ground are featured. In Part III, there are two chapters on recent developments to understand the detection and processing of volatile signals by plants. Plant electrophysiology and volatile uptake and conversion are the key concepts explored, which complement and add to the ecology of plant–plant interactions covered in

Part II. Some chapters in the book, particularly Chaps. 4, 8 and 12, provide detailed information on current methodologies and offer perspectives on future applications to advance the field of chemical ecology and further elucidate the chemical language of plants. We finish with a synthesis of the key findings within the book and some further ideas for future research directions.

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Part I

Plant Volatiles: Complexity of Chemical Messages

Chapter 1
Temporal Dynamics of Plant Volatiles: Mechanistic Bases and Functional Consequences

Meredith C. Schuman, Henrique A. Valim, and Youngsung Joo

Abstract Plant volatiles comprise thousands of low-molecular weight, hydrophobic molecules that are classified as ‘secondary’ (specialized) metabolites, but are closely related to ‘primary’ (general) metabolites such as fatty acids, amino acids, sterols and carotenoids. In addition to having important physiological functions, these specialized small molecules have a large influence on plants’ ecological interactions. By emitting particular blends of volatiles, plants can provide detailed information about their current physiological and ecological states and even manipulate other organisms. In fact, the timing of volatile biosynthesis and emission may be as critical to function as the amount and composition of volatile blends. Here, we critically review the known and hypothesized effects of phenological changes in plant volatile emission, their regulation and importance for function.

1.1 Introduction

In life, time is of the essence. This is no mere cliché but rather an ecologically sound generalization: nearly all multicellular eukaryotes, as well as photosynthetic prokaryotes—the cyanobacteria—possess internal clocks that permit the coordination of their metabolism and activity with diurnal cycles of abiotic factors such as light,

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temperature and moisture (Bell-Pedersen et al. 2005; Yerushalmi and Green 2009). Perhaps as an emergent property of the coordination between individual organisms and abiotic cycles, circadian clocks also allow organisms to coordinate with each other's diurnal activity patterns [Wang et al. 2011; Goodspeed et al. 2012 (but see Jander 2012); Zhang et al. 2013]. Ontogenetic events also determine timing and prioritization of phenotypes due to, e.g. developmental necessity, adaptation to environmental changes or the transition from vegetative growth to reproduction. And while timing is important in general for ecology, it is crucial in the production and emission of plant volatiles.

1.1.1 A Brief Introduction to Plant Volatiles

Volatile compounds are small molecules (generally <300 Da) which are sufficiently lightweight and low polarity to have high vapour pressures under normal environmental conditions (reviewed in Dudareva et al. 2006; Baldwin 2010). These molecules may come from any of several biosynthetic pathways that are closely linked to pathways or products of general metabolism, i.e. fats and other lipids, amino acids and proteins (reviewed in Dudareva et al. 2006; Goff and Klee 2006; Baldwin 2010). The biosynthetic classes of plant volatiles and their known structures and functions are described in detail in Table 1.1, and example structures are shown in Fig. 1.1. In addition to the compounds shown, large amounts of methanol (μmol min^{-1}) can be produced from the demethylation of pectin in cell walls, and this process is induced by wounding and herbivory (von Dahl et al. 2006).

Plant volatiles have important roles within plant tissues in physiology, signalling and defence. When emitted through the cuticle, stomata or wounded tissue or from specialized structures (reviewed in Widhalm et al. 2015), they may be perceived by a host of other organisms as well as by remote parts of the plant (Heil and Silva Bueno 2007; reviewed in Baldwin 2010; Dicke and Baldwin 2010). The composition of volatile blends can convey detailed information about the physiological and ecological status of plants—such as the presence of open flowers, attack by herbivores, infection by microbes and production of ripe fruit—which may be used by microbes, animals and other plants, both detrimental and beneficial (reviewed in Dicke and Baldwin 2010). The timing of both production and emission of floral and vegetative volatiles is thus essential to their function in within-plant signalling, as well as in orchestrating interactions with other organisms, and may determine their potential for exploitation by enemies.

In this chapter, we provide an overview of what is currently known about the importance of timing in plant volatile biosynthesis and emission; the roles of plant volatiles we briefly refer to in this overview are elaborated throughout this book. The word importance has no precise definition in biology. We use importance to refer to the biological reasons underlying timing: why and how the production and emission of plant volatiles is timed in particular ways. Biologically, why and how can be precisely defined as distinct levels of analysis at which biological

Table 1.1 Biosynthetic classes of plant volatiles and their biosynthesis, proposed functions and structural variety

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)^{a}	Number of known structures
Fatty acid derivatives	Jasmonates	Jasmonates are oxylipins synthesized from 16:3 and 18:3 fatty acids dioxygenated at C13 by 13-lipoxygenase (13-LOX) (Wasternack 2007)	Methyl jasmonate is among the main components of the scent of the jasmine flower, where it was first discovered (Demole et al. 1962). It is a volatile plant hormone which may be involved in plant–plant interactions (Karban et al. 2000; Preston et al. 2001; Kessler et al. 2006), as may the volatile (Z)-jasmone (Birkett et al. 2000)	Methyl jasmonate 302.9 (*Z*)-Jasmone 292	Four stereoisomers
	Green leaf volatiles	GLVs are synthesized via the cleavage of 13-LOX products by hydroperoxide lyase (HPL) to yield hexan-1-al (from 18:2 fatty acids) or (*Z*)-3-hexen-1-al (18:3). (*Z*)-3-Hexen-1-al can be isomerized to (*E*)-2-hexen-1-al spontaneously or by an isomerase; the hexenals can be converted to alcohols by alcohol dehydrogenases, and the alcohols can be esterified (Matsui et al. 2006)	Green leaf volatiles make up the ‘cut grass’ smell typical of wounded plant tissue (Hatanaka et al. 1987), contribute to the odour of fruits and flowers(Dudareva et al. 2006) and are emitted from roots and are important recognition and flavour components for animal consumers (Halitschke et al. 2004). GLVs may contribute to plant defence as antimicrobials (Deng	(*Z*)-3-Hexen-1-al 127.3 (*Z*)-3-Hexen-1-ol 156.5 (*Z*)-3-Hexen-1-yl acetate 174.2	At least 32 known from plants: four aldehydes (hexan-1-al, (*Z*)-3-hexen-1-al, (*E*)-2-hexen-1-al, and (*E*)-3-hexen-1-al) which provide substrate for four alcohols and at least 24 esters (acetates, propionates, butyrates, isobutyrates, valerates and isovalerates)

(continued)

Table 1.1 (continued)

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[a]	Number of known structures
			et al. 1993) or antifungals (Shiojiri et al. 2006a), direct (Vancanneyt et al. 2001) and indirect (Shiojiri et al. 2006a) anti-herbivore defences, and between (Baldwin et al. 2006; Paschold et al. 2006)-and within-plant (Frost et al. 2008) cues or signals		
	Nine-carbon volatile aldehydes, alcohols and esters	Nine-carbon derivatives are synthesized from 9-lipoxygenase (9-LOX) products of 18:2 and 18:3 fatty acids cleaved at the ninth carbon by HPL and, like GLVs, include aldehydes, alcohols and esters. Some HPLs specifically cleave 9- or 13-hydroperoxides, whereas others cleave both 9- and 13-hydroperoxides. 9-HPL products from 18:2 fatty acids contain one double bond, and those from 18:3 fatty	9-HPL products and their derivatives are odour and flavour components of flowers and fruits (Vancanneyt et al. 2001) and may be involved in seed development in almond (Mita et al. 2005). 9C aldehydes have antifungal properties (Matsui 2006)	(*E*,*E*)-3,6-Nonadienal 201.8 (*E*,*E*)-3,6-Nonadienol 214.7 (*E*,*E*)-3,6-Nonadienyl acetate 247.4	At least 15: five aldehydes which can be converted to five alcohols, which can be esterified; only the acetate esters are well represented in literature

		acids contain two double bonds (De Domenico et al. 2007)			
Terpenoids	Terpene hydrocarbons: Most volatile terpenoids have a five- (hemiterpene), ten- (monoterpene), or 15- (sesquiterpene) carbon skeleton	Terpene hydrocarbons are synthesized from the 5-carbon precursor isopentyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP) produced via one of two pathways in plants: the 2-C-methyl-derythritol 4-phosphate (MEP) pathway in plastids or the mevalonic acid (MVA) pathway in the cytosol. Generally, hemiterpenes and monoterpenes are synthesized in the plastid and sesquiterpenes in the cytosol; some sesquiterpenes may be synthesized in the mitochondrion from cytosolic substrate (Rodríguez-Concepción 2006; Kappers et al. 2005). Emission is usually light-dependent (Lerdau and Gray 2003)	Terpene hydrocarbons are components of flower, fruit, green tissue and root odours. Many are allelopathic (Junya Mizutani 1999) or may act in plant defence as antimicrobials or antifungals (Cowan 1999; Khosla and Keasling 2003), direct (cytochrome P450-inducing) (Brattsten 1983) and indirect (Degenhardt et al. 2003) antiherbivore defences or attract pollinators (Schiestl 2010). Most react with atmospheric ozone (Calogirou 1999) and could be involved in plant oxidative stress responses (Vickers et al. 2009)	Isoprene 34.1 Monoterpenes ca. 140–180 (Harborne 1973) Sesquiterpenes: >200 (Harborne 1973)	Isoprene is the only hemiterpene. At least 1000 different mono- and approximately 5000 different sesquiterpenes are known (Seigler 2008). Most are mono- or polycyclic
	Some terpenoids have an irregular number of carbons (8–18); these are called homoterpenes or	The homoterpene trans, trans-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E*,*E*)-	(*E*,*E*)-TMTT and (*E*)-DMNT are herbivore-induced volatiles in many plants and can attract	(*E*,*E*)-TMTT 293.2 (*E*)-DMNT 195.6	(*E*,*E*)-TMTT and (*E*)-DMNT are the only homoterpenes known to be widespread in plants.

(continued)

Table 1.1 (continued)

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[a]	Number of known structures
	apocarotenoids depending on their origin and are derived from the cleavage of larger terpenoids	TMTT] (C16) is derived from the diterpene geranyllinalool (C20) in the plastid (Herde et al. 2008), and the homoterpene trans-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT] (C11) is derived from the sesquiterpene (3S)-trans-nerolidol (C15) in the cytosol (Boland and Gäbler 1989) by oxidative degradation, possibly catalyzed by cytochrome P450 enzymes (Dudareva et al. 2006). Apocarotenoids (C8-C18) are cleaved from carotenoids in the plastid by carotenoid cleavage oxygenases (CCOs) (Auldridge et al. 2006; Walter et al. 2010)	parasitoids and predators to plants with feeding herbivores (Dudareva et al. 2006). Apocarotenoids are flavour and odour components of flowers, fruit and green tissue (Camara and Bouvier 2004). In flowers, apocarotenoids increase apparency both to pollinators and predators and may be attractive or repellent; in fruit, they are associated with ripening (Bouvier et al. 2005). Some have antifungal properties (Maffei 2010)		Additionally, three different homoterpenes are emitted from elm leaves following oviposition by the elm leaf beetle *Xanthogaleruca* luteola (Wegener and Schulz 2002), and other structures are known in insects
	Oxidized terpenes and derivatives	Terpenoid hydrocarbons may be further modified by, e.g. cytochrome P450 enzymes, and the	Oxidized terpenes and derivatives are also components of flower, fruit, green tissue and root	Boiling points are higher than the corresponding terpene hydrocarbons	Thousands

		products may be oxidized by dehydrogenases, esterified by acyltransferases, or reduced. Some terpene synthase enzymes incorporate a molecule of CO_2 to produce oxidized terpenoids as their initial product (Dudareva et al. 2006). Terpene alcohols may also be glycosylated, but the glycosides are not volatile	odours (Dudareva et al. 2004). They have similar ecological and physiological roles to those of terpene hydrocarbons but are more often directly toxic (Khosla and Keasling 2003)		
Phenylpropanoids and benzenoids	Acid, aldehyde and alcohol derivatives of L-phenylalanine	L-phenylalanine is converted to trans-cinnamic acid via L-phenylalanine ammonia lyase (PAL). Further conversion of trans-cinnamic acid to other phenylpropanoids is shared with the lignin biosynthetic pathway through the steps of monolignol biosynthesis. Benzenoids originate from the same biosynthetic pathway, but the side chain of transcinnamic acid is enzymatically shortened	Common in floral scents (Vogt 2010); source of pungent flavour in black pepper and chili peppers (capsaicinoids). Methyl salicylate is a component of an herbivore-induced blend attractive to some predators and parasitoids (Van Poecke et al. 2001; Ament et al. 2004)	ca. 180–325 (Oyama-Okubo et al. 2005)	ca. 20 % of all known plant volatiles (Qualley and Dudareva 2008)

(continued)

Table 1.1 (continued)

Class	Compounds	Biosynthesis	Functions	Volatility (BP °C)[a]	Number of known structures
		by two carbons. L-phenylalanine derivatives with a C2 side chain compete with phenylpropanoids and benzenoids for substrate and are synthesized via different pathways (Dudareva et al. 2006)			
Amino acids and derivatives	Acids, aldehydes, alcohols, esters, nitrogen- and sulphur-containing VOCs derived from amino acids other than L-phenylalanine	Amino acids are deaminated or transaminated to form α-keto acids, which are carboxylated and may subsequently be reduced, oxidated or esterified. Amino acids may also be precursors for acyl coA molecules used in esterification reactions catalyzed by alcohol acyltransferases (Dudareva et al. 2006). Ethylene is derived from methionine and thus belongs in this category	Branched-chain amino acid (Leu, Ile, Val) derivatives are common in fruit. Amino-acid derived esters are found in flowers and fruits (Dudareva et al. 2006). Putrid sulphur-containing compounds, likely derived from methioinine (Dudareva et al. 2006), may serve as direct defences (Berkov et al. 2000)	Ethylene −103.7 3-Methylbutan-2-ol 113.6 Butyl acetate 126.6	

[a] Royal Society of Chemistry. acc. July 2010. ChemSpider: http://www.chemspider.com/

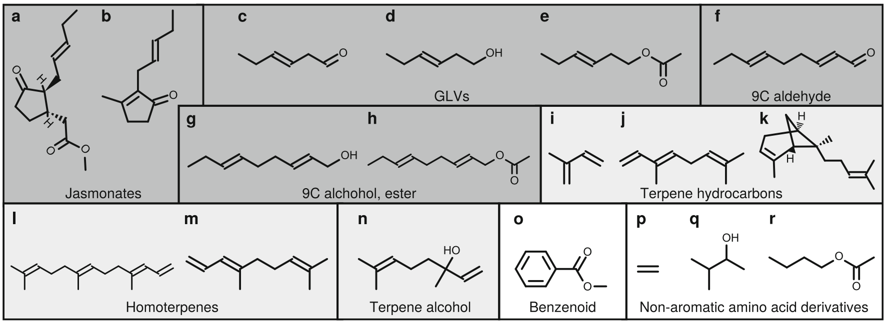

Fig. 1.1 Example structures from the biosynthetic classes of plant volatiles described in Table 1.1: the jasmonates methyl jasmonate (**a**) and (*Z*)-jasmone (**b**); GLVs (*Z*)-3-hexen-1-al (**c**), (*Z*)-3-hexen-1-ol (**d**) and (*Z*)-3-hexen-1-yl acetate (**e**); 9C compounds (*E*,*E*)-3,6-nonadienal (**f**), (*E*,*E*)-3,6-nonadienol (**g**) and (*E*,*E*)-3,6-nonadienyl acetate (**h**); terpene hydrocarbons isoprene (**i**), monoterpene β-ocimene (**j**) and sesquiterpene (*E*)-α-bergamotene (**k**); homoterpenes (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene [(*E*,*E*)-TMTT] (**l**) and (*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT] (**m**); monoterpene alcohol linalool (**n**); benzenoid methyl benzoate (**o**); and non-aromatic amino acid derivatives ethylene (**p**), 3-methylbutan-2-ol (**q**) and butyl acetate (**r**). Classes with the same type of biosynthetic precursors (fatty acids, IPP/DMAPP, amino acids) have identical background shading. Methanol, produced abundantly by demethylation of pectin in plant walls, is not shown

phenomena can be investigated: 'why' can be answered either in terms of functional outcomes or evolutionary history, and 'how' may be answered in terms of physiological or ontogenetic events (Tinbergen 1963; Sherman 1988).

Here, we focus on a functional level of analysis, including mechanistic and evolutionary dimensions when appropriate to provide a more complete biological picture. We begin from the assumption that observed traits are adaptive and reject this view only if evidence does not support it. There is abundant evidence against the alternative assumption that plant volatiles are waste products, released from plants more as a result of their physiochemical properties than of physiological mechanisms, which may opportunistically accrue functions (Niinemets et al. 2004; Peñuelas and Llusià 2004; Rosenstiel et al. 2004). This is reminiscent of the more than 60-year-old theory that specialized plant metabolites generally are 'flotsam and jetsam on the metabolic beach', comprising waste products which may be opportunistically co-opted for functional roles, a theory which has not been supported for any plant specialized metabolite biosynthetically, physiologically, evolutionarily or functionally studied so far (Hartmann 2007). In fact, even the emission of plant volatiles through cuticles, formerly thought to occur passively, is likely to be actively regulated in order to avoid toxic concentrations of volatiles in membranes (Widhalm et al. 2015).

1.1.2 Why Are Plant Volatiles Synthesized and Emitted in Certain Tissues at Certain Times?

1.1.2.1 Function

In order to synchronize with abiotic and biotic factors, organisms need to have a sense of timing. Rhythmic behaviour can efficiently increase fitness by optimizing plant metabolism to abiotic circumstances dictated by the timing of sunrise and sunset (reviewed in Yerushalmi and Green 2009). Since different times of day bring different abiotic conditions and thus biotic communities, each plant has its own ‘specific timing’ to maximize its fitness (Raguso et al. 2003). This is realized not only in visually apparent behaviours like flower opening and leaf movement but also in the emission of plant volatiles, which often show distinct diurnal or nocturnal patterns in different tissues. Plants produce different amounts and combinations of volatiles over time, and these volatile blends have different physiological and ecological functions including:

- Within-plant signalling, which also leads to ‘eavesdropping’ on other plants (Baldwin and Schultz 1983; Rhoades 1983; Heil and Silva Bueno 2007; reviewed in Heil and Karban 2010)
- Pollinator attraction (Kessler et al. 2008)
- Seed dispersal (Bolen and Green 1997; Luft et al. 2003; Goff and Klee 2006; Klee and Giovannoni 2011)
- Deterrence of herbivores, although they can also be co-opted by herbivores as host location cues (Kessler and Baldwin 2001; De Moraes et al. 2001; Halitschke et al. 2008; reviewed in Bruce et al. 2005) and feeding stimulants (Halitschke et al. 2004; Meldau et al. 2009)
- Attraction of predators and parasitoids to defend against herbivores (Dicke 1986; De Moraes et al. 1998; Kessler and Baldwin 2001; Rasmann et al. 2005; Schuman et al. 2012; reviewed in Dicke and Baldwin 2010; McCormick et al. 2012)
- Mediating interactions with microbes (reviewed in Junker and Tholl 2013)
- Allelopathic inhibition of neighbours (Inderjit et al. 2009; reviewed in Glinwood et al. 2011)
- Tolerance of abiotic stress (reviewed in Holopainen and Gershenzon 2010)

These roles can be viewed on a spectrum from internal to external, in terms of whether plant volatiles act within or between plant cells, or after emission into the environment, and are usually localized to particular tissues (Fig. 1.2).

1.1.2.2 Evolution

From the evolutionary perspective, the answer to ‘why’ plants synthesize and emit particular volatiles, at particular times and from particular tissues or structures, is the evolutionary trajectory resulting in the currently observed phenotype.

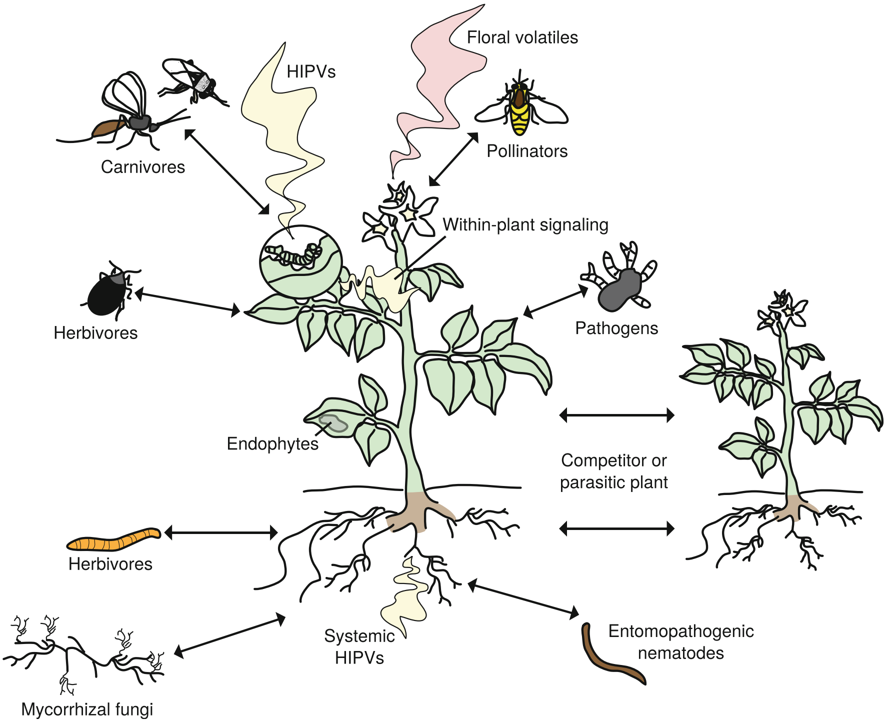

Fig. 1.2 Functional roles of plant volatiles. Modified with permission from Dicke and Baldwin (2010)

Evolutionary analyses usually study the appearance and mutation of biosynthetic enzymes (reviewed in Pichersky et al. 2006), though they may also focus on structures for biosynthesis, storage or emission (reviewed in Lange 2015). The enzymes of GLV and jasmonate biosynthesis are ubiquitous in plants, originating in the green algae (reviewed in Andreou et al. 2009; Lange 2015), and the mevalonate and methylerythritol phosphate pathways providing substrate for terpenoid volatiles can be found in bacteria (reviewed in Rodríguez-Concepción et al. 2013; Lange 2015). In contrast, many volatile end products are limited to particular plant lineages, indicating rapid diversification of downstream biosynthetic enzymes (reviewed in Pichersky et al. 2006). However volatiles from most biosynthetic classes are emitted from vegetative, floral and root tissues in higher plants (reviewed in Dudareva et al. 2004) (although roots are more difficult to analyse and thus less well studied).

The enzymes of plant volatile biosynthesis are derived from general metabolism. For example, the diverse family of terpene synthases is derived from an ancestral ent-Kaurene synthase (Trapp and Croteau 2001, reviewed in Chen et al. 2011). The enzymes of jasmonate and GLV biosynthesis carry out the controlled degradation of reactive free fatty acids; substrate for phenylpropanoid and benzenoid volatiles comes from intermediate products of lignin biosynthesis, and other volatiles derive from salvage and degradation pathways of amino acids via α-keto acids (reviewed in Dudareva et al. 2006). Thus volatile metabolites are more or less closely linked to the dynamics, regulation and rhythms of general metabolism.

1.1.3 How Is the Induced, Diurnal and Ontogenetic Timing of Volatile Biosynthesis and Emission Regulated?

The roles of physiology and ontogeny in plant volatile synthesis and emission are depicted in Fig. 1.3.

1.1.3.1 Physiology

Physiology provides the immediate mechanisms. The close relationship of plant volatiles to general metabolic pathways dictates precursor availability according to diurnal or circadian control (Pokhilko et al. 2015), hormonal signalling and flux channelling (reviewed in Dudareva et al. 2006; Nagegowda 2010; Vranová et al. 2012). Direct volatile biosynthetic enzymes may be regulated by any or all of these cues, and many of the best-studied examples are of herbivory-induced volatile emission (reviewed in Howe and Jander 2008). Emission, like biosynthesis, is also tightly regulated—even the diffusion of plant volatiles across membranes and cuticles is likely to be an actively regulated process (Widhalm et al. 2015). Other than diffusion, emission may occur through stomata (Seidl-Adams et al. 2014), wounds (De Domenico et al. 2007), or specialized tissues such as flowers (reviewed in Muhlemann et al. 2014) or glandular trichomes (e.g. Schuurink 2007; but see Hare 2007). Emission rates are influenced by internal signalling (reviewed in Howe and Jander 2008) and other wounding-related processes (e.g. De Domenico et al. 2007) as well as temperature, humidity and light levels, both due to direct physical effects on volatility and—likely more importantly—to the effects of these factors on the physiological mechanisms of emission (e.g. Grote et al. 2014).

1.1.3.2 Ontogeny

Ontogeny describes the second mechanistic or the ‘how’ level at which we may investigate the regulation of plant volatile biosynthesis and emission, comprising in part the regulatory physiological system of each life stage and tissue of the plant. To a large extent, however, ontogenetic changes may accompany the development of new tissues and transitions to different life stages, e.g. from vegetative to reproductive (Diezel et al. 2011). Perhaps due in part to overlap in the emission profiles

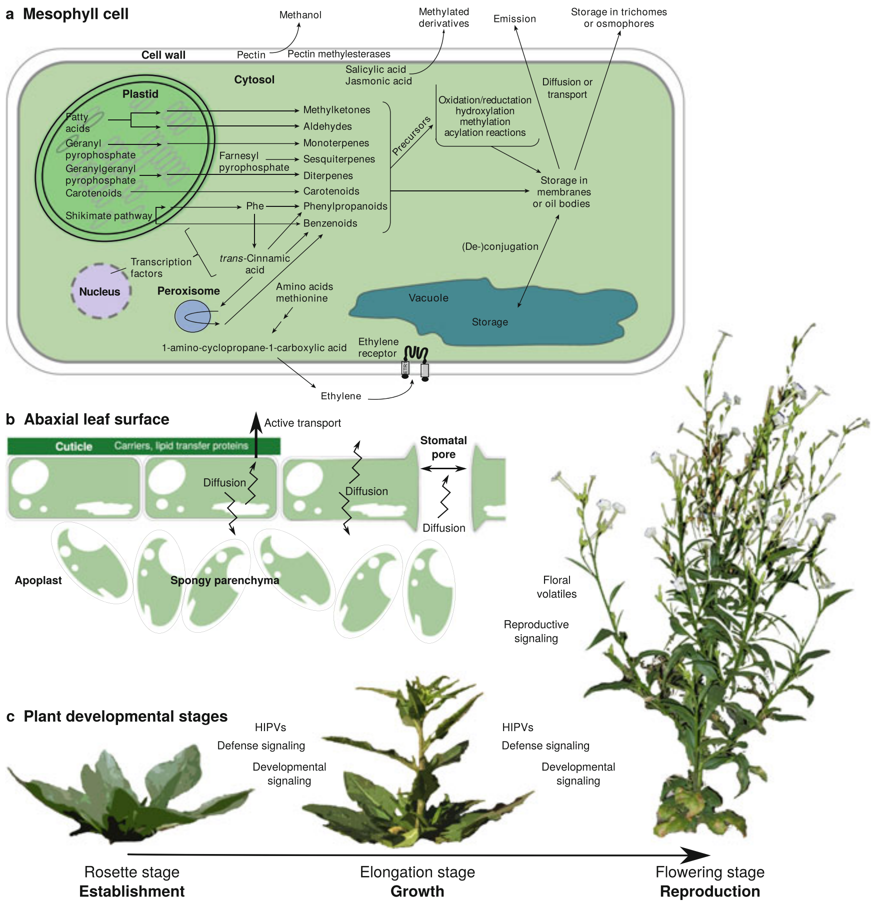

Fig. 1.3 Mechanisms of plant volatile biosynthesis and emission. (**a**) Physiological mechanisms, modified with permission from Baldwin (2010). (**b**) Depiction of possible paths for volatiles—once synthesized, released from conjugates or intracellular storage—to diffuse (over cell walls, through stomata) or be actively transported (through cuticles) to the headspace, using the abaxial leaf surface as an example. (**c**) Vegetative and reproductive stages of the ecological model plant *Nicotiana attenuata* visualized as changes to shoots and changing roles of plant volatiles. Roots and seeds are not shown as less is known about the structural changes or dynamic volatile emissions of these tissues. Plants modified from Schuman et al. (2014)

of different tissues or to the hormonal signalling functions of some volatiles, vegetative volatiles may interfere with the function of floral volatiles and vice versa (von Dahl et al. 2007; Diezel et al. 2011; Kessler et al. 2011; Desurmont et al. 2015). Ontogenetic changes in volatile emission may serve to avoid such interference. Furthermore, both within tissues and across life stages, the development of specialized cells and tissues may be required for the biosynthesis and emission of some volatiles, placing physiological limitations on ontogenetic timing (e.g. Bate et al. 1998; reviewed in Dudareva et al. 2004; Rodríguez-Concepción et al. 2013).

1.2 The Importance of Timing in Plant Volatile Emission

In the following sections, we describe how several well-studied phenomena mediated by plant volatiles can be understood on the timescales of induction, diurnal and circadian rhythms and changes over ontogeny. We focus on functions but also address their regulation.

1.2.1 Plant Volatiles as Induced Defences and Defence Signals

It is well known that biosynthesis and emission of specific plant volatiles are induced by stress in every higher plant studied so far (reviewed in Holopainen and Gershenzon 2010), resulting in predictable volatile blends which can serve as reliable cues or signals: cues benefit the receiver with an unspecified effect on the sender, while signals benefit the sender and the receiver, with ‘benefit’ defined biologically as increase in Darwinian fitness (Greenfield 2002; Allison and Hare 2009). Stress-induced plant volatile blends may be highly specific to particular plant genotypes (e.g. Wu et al. 2008; Delphia et al. 2009; Schuman et al. 2009; reviewed in Wu and Baldwin 2010) and stress events (reviewed in Holopainen and Gershenzon 2010), including specificity to particular herbivores (reviewed in McCormick et al. 2012) and numbers of herbivores (e.g. Shiojiri et al. 2010). The ubiquity and specificity of stress-induced plant volatile emission indicates the importance of these compounds in structuring ecological communities, from effects on pollinators, herbivores, predators and microbes, to impact on global climate (reviewed in Kessler and Halitschke 2007; Arneth and Niinemets 2010; Dicke and Baldwin 2010; Holopainen and Gershenzon 2010). Herbivore-induced plant volatiles (HIPVs) are among the best-studied induced phenomena in the field of plant–herbivore interactions and have served as highly specific phenotypic read-outs in studies of herbivore elicitors (reviewed in Howe and Jander 2008).

1.2.1.1 Functions of Herbivore-Induced Plant Volatiles (HIPVs)

As semiochemicals, HIPVs can attract parasitoids of herbivores (De Moraes et al. 1998) and insectivorous carnivores (Dicke 1986; Kessler and Baldwin 2001) and deter herbivore oviposition (Kessler and Baldwin 2001; De Moraes et al. 2001), all of which may enhance plant fitness by increasing the apparency of herbivores to predators (Feeny 1976). However, HIPVs and other plant volatiles also act as host location cues for herbivores (e.g. Halitschke et al. 2008; reviewed in Bruce et al. 2005; Dicke and Baldwin 2010). In *N. attenuata*, the release of HIPVs reduces herbivore loads by 50 % or more on the emitting plants (Kessler and Baldwin 2001; Allmann and Baldwin 2010; Schuman et al. 2012) allowing GLV-emitting plants to produce twice as many flowers, buds and seed capsules as non-emitters in the presence of predators (Schuman et al. 2012). HIPVs can also contribute to defence signalling within plants, for example, eliciting the production of hormones and gene transcripts involved in defence and eliciting or priming defence traits (Heil and Silva Bueno 2007; Frost et al. 2008; reviewed in Heil and Karban 2010). Likely as a side effect of their ability to respond to their own HIPVs, plants can also elicit or prime defence responses after ‘eavesdropping’ on HIPVs from neighbours (reviewed in Baldwin et al. 2006; Heil and Karban 2010; Scala et al. 2013; and see Chap. 7); responses may be specific to plant genotype and interaction (e.g. Li et al. 2012). Neighbour volatiles have also been shown to alter biomass allocation and growth in barley (Ninkovic 2003; Kegge et al. 2015).

1.2.1.2 Regulation of HIPVs

HIPV emission is regulated by the same signalling systems that elicit other induced defence responses in plants (Fig. 1.3). For example, it has been shown that different elicitors in the regurgitant of *Manduca sexta* (tobacco hornworm) larvae elicit specific HIPVs in *Nicotiana attenuata* (wild coyote tobacco) (Gaquerel et al. 2009). Furthermore, in *N. attenuata*, mitogen-activated protein kinase (MAPK) signalling (Meldau et al. 2009), jasmonate signalling (Halitschke and Baldwin 2003; Schuman et al. 2009; Oh et al. 2012; Woldemariam et al. 2012, 2013), abscisic acid signalling (Dinh et al. 2013) and WRKY transcription factors (Skibbe et al. 2008) all have been shown to regulate HIPV emission. Some isoprenoids have been shown to react with and quench reactive oxygen species (ROS) (reviewed in Vickers et al. 2009; Holopainen and Gershenzon 2010). ROS are a component of early signalling events that induce stress hormone signalling, including abscisic acid and jasmonate signalling (reviewed in Maffei et al. 2007; Wu and Baldwin 2010), and are likely to be involved in the induction of HIPVs and other stress-responsive volatiles.

Interestingly, in *Arabidopsis* (*Arabidopsis thaliana*), it has been shown that HIPV emission is dependent on jasmonates but not on the known active form jasmonoyl isoleucine (JA-Ile) (Fonseca et al. 2009), indicating that a different jasmonate may activate genes controlling HIPV emission, while JA-Ile regulates other defence responses (Van Poecke and Dicke 2003; Wang et al. 2008). In *Phaseolus lunatus* (lima bean), synthetic JA-Ile analogues similar in structure with coronatine, the highly active jasmonate mimic produced by the biotrophic plant pathogen *Pseudomonas syringae*, strongly elicit HIPV biosynthesis as long as the carbonyl group on the 5-membered ring is intact and regardless of modifications to the amino acid moiety (Krumm et al. 1995). Application of methyl jasmonate has also been shown to elicit volatile emission in other wild and domesticated plants including *Solanum peruvianum* (Peruvian wild tomato) (Kessler et al. 2011), *Datura wrightii* (sacred Datura) (Hare 2007), *N. attenuata* (Halitschke et al. 2000), *P. lunatus* (Ozawa et al. 2000) and *Gossypium hirsutum* L. (cotton) (Rodriguez-Saona et al. 2001). The volatile emission elicited by jasmonate treatment often partially, but not fully, overlaps with the profiles elicited by feeding of different herbivores (e.g. Dicke et al. 1999; Kessler and Baldwin 2001).

1.2.2 Diurnal and Circadian Rhythms of Stress-Induced Volatiles

Volatiles are generally emitted beginning from seconds to hours following induction, and the emission induced by a single event may last as little as minutes or as long as days (see, e.g. Loughrin et al. 1994; von Dahl et al. 2006; Allmann and Baldwin 2010; Schaub et al. 2010; Danner et al. 2012; Jardine et al. 2012; Kallenbach et al. 2014). Longer emission cycles may display diurnal rhythms (e.g. Loughrin et al. 1994; Arimura et al. 2008) (Fig. 1.4), which may allow plants to synchronize volatile emission temporally with biotic factors, e.g. herbivores, predators and parasitoids. This could increase plant fitness, because many insects also have their own predictable rhythmic behaviour. For example, circadian-regulated jasmonate accumulation can enhance plant resistance to herbivores with synchronized feeding activity (Goodspeed et al. 2012; but see Jander 2012). Also, nocturnal volatiles produced by host plants repelled oviposition of nocturnal moths (De Moraes et al. 2001; Allmann et al. 2013), and determined the behaviour of *Mythimna separata* (Northern armyworm) larvae (Shiojiri et al. 2006b).

1.2.2.1 Rhythmic Volatile Emission in Response to Biotic and Abiotic Stress

Most diurnal plant behaviours are synchronized to daily rhythms of abiotic factors like light and temperature, denoted ‘zeitgeber’ (time givers). In particular, many isoprenoids and terpenoids show strong diurnal rhythms because 75 % of carbon for isoprenoid synthesis originates from photosynthesis, and the methylerythritol phosphate (MEP) pathway has strong diurnal and circadian rhythms (Schnitzler et al. 2004; Dudareva et al. 2005; Pokhilko et al. 2015). In many tree species, monoterpene production positively correlates with light intensity and temperature (Tingey et al. 1980; Harley et al. 2014; Jardine et al. 2015). It should be noted that the lower volatility of sesquiterpenes (Table 1.1) can lead to experimental artefacts, e.g. resulting from their temperature-dependent adsorption to and re-release from collection cuvettes, which can obfuscate emission patterns (Schaub et al. 2010). However herbivore-induced emission of the monoterpene β-ocimene in *P. lunatus*

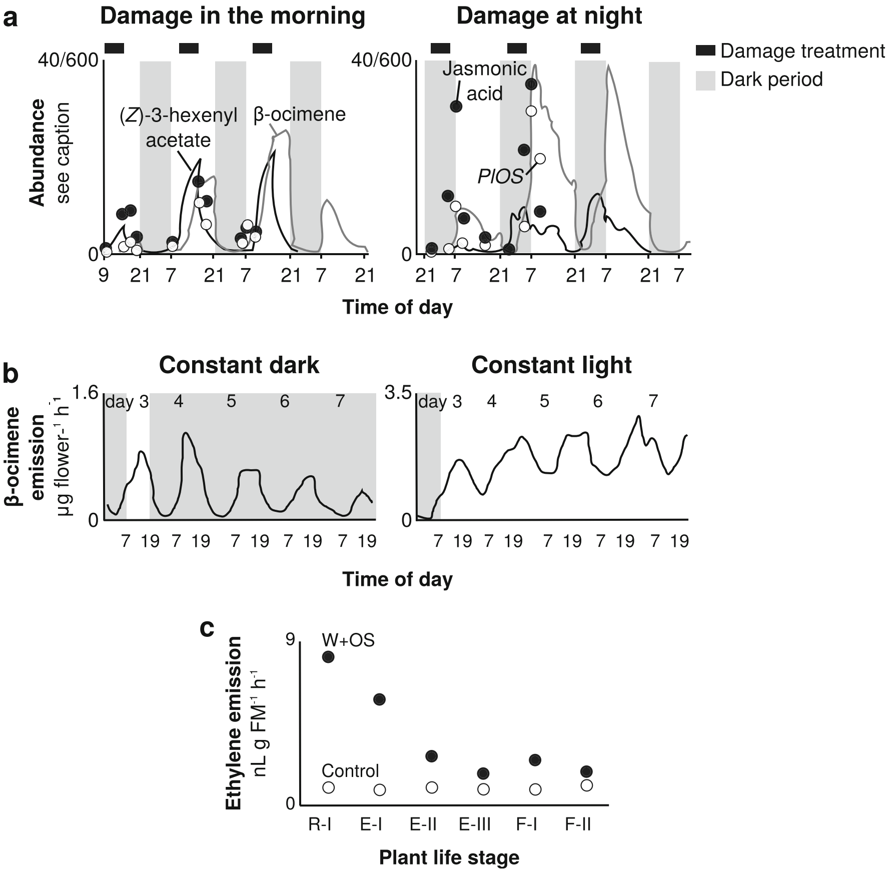

Fig. 1.4 Examples of phenological changes in plant volatile emission. (**a**) The monoterpene β-ocimene is emitted from *Phaseolus lunatus* leaves during light periods following simulated herbivore damage (MecWorm treatment), while the GLV (*Z*)-3-hexenyl acetate is emitted immediately upon damage regardless of light or dark period (volatiles shown on the scale of 0–40 ng g FM^{-1} min^{-1}). Jasmonic acid and *PlOS* (β-ocimene synthase) transcript accumulation reflect dynamics of β-ocimene emission (jasmonic acid scale: 0–600 ng g FM^{-1}, *PlOS* transcripts relative to *PlACT1* transcripts). Scales in the *left* and *right panel* are the same, indicating that jasmonate accumulation and β-ocimene emission are greater after night-time damage. Drawn from data in Fig. 3 of (Arimura et al. 2008). (**b**) Circadian oscillation in the emission of β-ocimene from flowers of *Antirrhinum majus* (snapdragon) cv Maryland true pink. Overall, more β-ocimene is emitted under constant light conditions (note difference in scale between *left* and *right panels*). Drawn from data in Fig. 2 of (Dudareva et al. 2003). (**c**) Ethylene emission induced after treatment of mature; non-senescent *Nicotiana attenuata* leaves with mock herbivory (wounding and *Manduca sexta* oral secretions, W + OS) is attenuated as plants age; control, untreated leaf at same position. Stages: *R-I* rosette; *E-I* beginning elongation, first inflorescence visible but not fully developed; *E-II and E-III* elongated, consecutive days, buds develop rapidly but do not yet open; *F-I* first corolla elongation; *F-II* first fully opened flowers. Drawn from Fig. 1 in (Diezel et al. 2011)

leaves showed a diurnal rhythm, while emission levels strongly decreased under artificial dark treatment (Arimura et al. 2008) (Fig. 1.4). Although many volatile emission rhythms strongly diminish or disappear without environmental cues, some volatiles retain oscillation under free-running conditions, demonstrating circadian regulation (Hsu and Harmer 2013). Isoprene emission, for example, has a strong circadian rhythm, presumably to protect plants from heat stress and maintain photosynthesis and also to mitigate the effects of oxidative stress in response to abiotic stress-mediated ROS production (reviewed in Sharkey et al. 2008; Loreto and Schnitzler 2010). Stress-induced plant volatile emission and its responsiveness to diurnal and seasonal zeitgeber contribute significantly to atmospheric pollution and global climate (Arneth and Niinemets 2010).

1.2.2.2 Are There Examples of Circadian Regulation of HIPVs?

So far no leaf HIPVs have been reported to have circadian-regulated emissions. Perhaps synchronization via the circadian clock is more common either when dictated by abiotic factors as discussed above or in mutualistic interactions. The evolutionary strategy between plants and herbivores is a diffuse arms race: each side responds to selection pressure for counter-adaptation from the other side (Fox 1981). For example, plants are thought to diversify their production of defensive metabolites in response to herbivore adaptation to older defences (Speed et al. 2015). So if plants have developed rhythmic traits to synchronize with herbivore behaviour, the herbivore may experience selection pressure to change the behaviour and escape the synchronization. Thus we would predict it is uncommon to find cases of circadian-mediated synchronization between HIPVs and herbivore activity, e.g. as direct defence responses. However, if two species have mutualistic interactions, both sides could benefit from synchronization, and this may be one way in which mutualistic interactions increase the stability of communities (Georgelin and Loeuille 2014). If there are cases of the circadian regulation of HIPVs, these might originate in physiological roles of these volatiles, if the resulting rhythms are also compatible with their defensive roles. For example, a rhythm dictated by the oxidative stress produced by photosynthesis may also be compatible with the activity patterns of certain herbivores or their natural enemies, or insects may learn to recognize typical, physiologically dictated and thus tightly conserved rhythms of plant volatile emission.

1.2.3 Circadian Timing of Floral Volatile Emission

It is perhaps not surprising that floral volatiles provide the best-known examples of robustly regulated, rhythmic plant volatile emission. Many flowering plants require assistance from pollinators to transfer pollen between flowers, and species with self-compatible flowers often nevertheless benefit from out-crossing mediated by pollinators (reviewed in Rosas-Guerrero et al. 2014). Flowers thus produce attractant volatiles for pollinators to enhance chances of out-crossing (Kessler et al. 2008). Many plants that require insect pollinators have evolved very specific floral traits for this purpose (reviewed in Raguso 2004). They also produce particular floral volatiles at specific times to synchronize with the activity of their pollinators (reviewed in Muhlemann et al. 2014). *N. attenuata* produces benzyl acetone during the night to attract nocturnal moths, *M. sexta* and *Hyles lineata* (whiteline sphinx) (Kessler et al. 2008). Whereas *Petunia* spp. (petunia) flowers mainly produce benzenoids to attract night pollinators (Hoballah et al. 2005); *Antirrhinum majus* (snapdragon) cv. Maryland true pink flowers emit larger amounts of methyl benzonate during the day to attract bees (Kolosova et al. 2001).

Circadian regulation has been shown for many floral volatiles. Bee-pollinated *A. majus* flowers produce the monoterpene myrcene, (*E*)-β-ocimene and linalool specifically during the day, a rhythm maintained under constant light conditions (Dudareva et al. 2003) (Fig. 1.4) and also emit more methyl benzoate during the day (Kolosova et al. 2001), while moth-pollinated *Nicotiana suaveolens* (Australian tobacco) and *Petunia cv Mitchell* flowers produce benzenoid volatile compounds at night, a rhythm maintained under constant dark conditions (Kolosova et al. 2001). Recently, evidence that the circadian clock directly regulates floral volatiles was reported in *Petunia hybrida* and *N. attenuata* (Yon et al. 2016; Fenske et al. 2015). Both studies showed that *late elongated hypocotyl* (LHY), a morning element of the circadian clock, is a main regulator in the peak timing of floral volatile emission. These findings suggest that the role of the circadian clock in flowers may be conserved in the Solanaceae. LHY may also transcriptionally regulate isoprene synthase to produce strong circadian rhythms of isoprene emission in grey poplar (Loivamäki et al. 2007). Circadian rhythms of plant volatiles are the consequence of circadian regulation of substrate flux and biosynthetic genes at transcriptional and enzymatic levels (Kolosova et al. 2001; Dudareva et al. 2005; Fenske et al. 2015; Pokhilko et al. 2015). In addition, circadian emission patterns of plant volatiles may also be influenced by the circadian regulation of emission mechanisms such as stomatal opening or transport through membranes (Lehmann and Or 2015; Widhalm et al. 2015) (Fig. 1.3).

1.2.4 Ontogenetic Changes in Plant Volatiles: Theory and Observation

1.2.4.1 Application of Plant Defence Theory to Ontogenetic Patterns

The composition of specialized metabolites in plants, including volatiles, varies not only in response to stress and diurnal events but also throughout lifecycles, across plant life histories and by type of herbivore pressure exerted on plants (Barton and Koricheva 2010). Plant defence theories have long sought to explain this variation with different degrees of success, and because many studies of variation in plant volatile emission are based on plant defence theory, it is important to mention them here. The most commonly employed have been optimal defence (OD) theory (McKey 1974; Rhoades and Cates 1976; McKey 1979; Rhoades 1979), apparency theory (Feeny 1976) and growth-differentiation balance (GDB) theory (Loomis 1932; Loomis 1953; Herms and Mattson 1992). Among these, GDB has been least effective in predicting ontogenetic changes described in literature. The view of ontogenetic changes in GDB is largely mechanistic but oversimplified, treating growth and differentiation as general processes at a level of understanding achieved in the first half of the twentieth century (Loomis 1932, 1953) and considering light and nutrient availability while ignoring other abiotic and biotic stimuli (Stamp 2003). As one study of defensive monoterpenes described, GDB is '. . .a source-driven model that does not, in its simplest form, consider changes in the need (demand) for growth or differentiation products' (Lerdau et al. 1994).

Apparency and OD theory have been more useful, despite not focusing on ontogeny, because many hypotheses related to the ontogenetic distribution of plant volatiles can also be posed as functional hypotheses. Further, apparency and OD theory provide some testable functional hypotheses, while the hypotheses posed by GDB theory mix mechanistic and functional levels of analysis and are thus not testable (Sherman 1988; Baldwin 1994; Stamp 2003). Functional studies largely treat ontogenetic stages as black boxes, with no exploration of the mechanistic background for differences which make ontogenetic stages distinctive, though not discrete. In contrast, the mechanistic literature is mostly restricted to developmental biology, though there are studies of the development of tissues, specialized storage and secretory structures which cross the boundary between developmental biology and the study of plant defence (reviewed in Dudareva et al. 2004), and studies of developmental regulation of biosynthetic enzymes in floral volatile emission (e.g. Pichersky et al. 1994; Bate et al. 1998; Dudareva et al. 2000). The literature on functions of plant volatiles over ontogeny is our focus here because we feel a critical discussion of the functional literature is lacking.

In a meta-analysis of 116 studies reporting ontogenetic patterns in plant defence traits across 153 plant species interacting with 30 herbivore species, Barton and Koricheva (2010) found that patterns over ontogeny in plant response variables (concentrations of secondary metabolites, measures of physical defence and tolerance) depended on plant life form, type of herbivore and type of response, and thus a generalization about patterns in defence over ontogeny could not be supported. The plant-age hypothesis (Bryant et al. 1992) explicitly applies predictions of OD theory to ontogenetic changes in plants, 'predicting that that extrinsic factors, namely, selection by herbivores, lead to high levels of defence in juveniles, followed by decreases as plants mature and become less susceptible to the fitness reductions of these attacks' (Barton and Koricheva 2010). Yet within a life stage, OD predicts that younger tissues should always induce higher direct and indirect defences, if they make a larger contribution to the plant fitness (reviewed in Meldau and Baldwin 2013). Of all tissues, then, reproductive tissues and seed capsules should be the best defended, representing the culmination of the plant's labours, as the rest of the plant senesces and concentrates metabolites in the reproductive organs (reviewed in Schippers et al. 2015). There is an apparent conflict between the predictions that ageing plants reduce defence and that reproductive tissues be well defended.

1.2.4.2 Functional Analyses of Ontogenetic Patterns, Informed by Mechanism

Tissue-based variations may be better explained by changes in plant volatile signalling functions, for example, in response to flowering and senescence (Stout 1996; Desurmont et al. 2015; Schippers et al. 2015), which are not predicted by plant defence theories. In field studies, inducibility of HIPVs has been observed to drastically decrease in reproductive-stage plants relative to plants undergoing only vegetative growth, for both *Glycine max* (soya bean) and *D. wrightii* (Rostás and Eggert 2008; Hare 2010). Critically, 'rejuvenation' by extensive trimming in *D. wrightii* (Hare 2010) partially restored HIPV production, indicating that the presence of reproductive tissues, not plant age, was responsible for HIPV attenuation. Changing 'functional priorities' for the volatiles in a plant's headspace may cause interference during flowering. In *B. rapa*, flowering also abolishes the elicitation of HIPVs from leaves, which causes a 20–30 % decrease in the attractiveness of plants to parasitoids in the face of infestation by the specialist cabbage butterfly (*Pieris brassicae*), likely with fitness consequences for the plant. Vegetative-stage plants perfumed with floral volatiles were also less attractive to parasitoids (Desurmont et al. 2015). In the other direction, HIPV emission induced by herbivory or methyl jasmonate application to *S. peruvianum* leaves reduced visitation of flowers by pollinating bees, reducing seed set. In contrast, removal of leaves from flowering plants (mimicking defoliation by herbivores) did not significantly reduce seed set unless at least 80 % of leaf tissue was removed (Kessler et al. 2011). Volatile emission from *G. max* after feeding by *Spodoptera frugiperda* (fall armyworm) caterpillars was also shown to be tenfold as great per gram of biomass in vegetative-stage plants, in comparison to reproductive-stage plants, and even within reproductive-stage plants, elicitation of HIPVs was 250-fold as great in leaves as in seed pods (Rostás and Eggert 2008). Differences in blend composition among leaves and seed pods could point to reasons why these large and perhaps unexpected differences exist, but these qualitative differences were not acknowledged or further explored. This is surprising given the body of literature covering the importance of blend composition for insect perception of volatile cues (reviewed in Bruce and Pickett 2011) and the emerging literature indicating its importance for plant responses to volatiles (reviewed in Ueda et al. 2012).

Likely depending on their roles in defence versus within-plant signalling, individual HIPVs often do not meet predictions of OD. In *N. attenuata*, mid-aged leaves (fully expanded, non-senescent) emitted the largest amounts of the sesquiterpene

(*E*)-α-bergamotene after induction (Halitschke et al. 2000), and (*E*)-α-bergamotene's inducibility does not decrease after flowering (Schuman et al. 2014). However in *G. max*, which produces both isomers of α-bergamotene, the inducibility of both decreases markedly to below detection after flowering; in the vegetative stage, *G. max* has also been shown to produce more total volatiles in younger than older leaves, as predicted by OD (Rostás and Eggert 2008). Whether the individual α-bergamotene isomers play different functional roles corresponding to differences in their regulation across species is unknown. The drastic decrease in herbivore-induced ethylene from leaves in flowering *N. attenuata* plants (Diezel et al. 2011) (Fig. 1.4) is posited to be due to ethylene's changing role from regulation of defence in the vegetative stage to regulation of pollen acceptance and flower senescence in the flowering stage (von Dahl et al. 2007; Bhattacharya and Baldwin 2012). In *N. attenuata*, emission of the moth-attracting floral volatile benzylacetone (Kessler et al. 2008) remains abundant from flowers up until the night of pollination, followed by a sharp reduction in benzylacetone emission and strong post-pollination ET bursts of up to ~300 nL g^{-1} h^{-1} that correlate strongly with pollination success based on the paternal genotype (Bhattacharya and Baldwin 2012). As for HIPVs in *D. wrightii* (Hare 2010), removal of inflorescences restores herbivore-inducible ethylene emission in *N. attenuata* (Diezel et al. 2011).

1.3 Conclusion

Volatile production and emission are dynamic characteristics of plants' responses to their environment over diurnal and ontogenetic time. Figure 1.5 provides a schematic integrating functions of plant volatiles over ecological and temporal scales. Tissue-based variations may lie at the heart of changing priorities for the plant, which allocates defences to different tissues based on specific 'goals' that change over ontogeny and include growth, defence and ultimately pollination and seed dispersal. Some plant volatiles may have co-evolved with specific ecological interaction partners like predators and pollinators, and the production of specific volatile blends may mirror the importance of particular interactions at that life stage. Plant volatiles also serve hormonal functions as the plant transitions through life stages. The unique roles of plant volatiles in defence and development as signals (for the plant) or cues (for other organisms) makes it difficult to apply plant defence theories (e.g. Ninkovic 2003; Pierik et al. 2004; Karban 2007; Rostás and Eggert 2008; Renne et al. 2014; Mirabella et al. 2015), which in any case often produce conflicting predictions (compare for example Barton and Koricheva 2010; and Meldau and Baldwin 2013). We suggest that further research is best guided by attempts to understand these complex phenomena at their functional, evolutionary, physiological and ontogenetic levels.

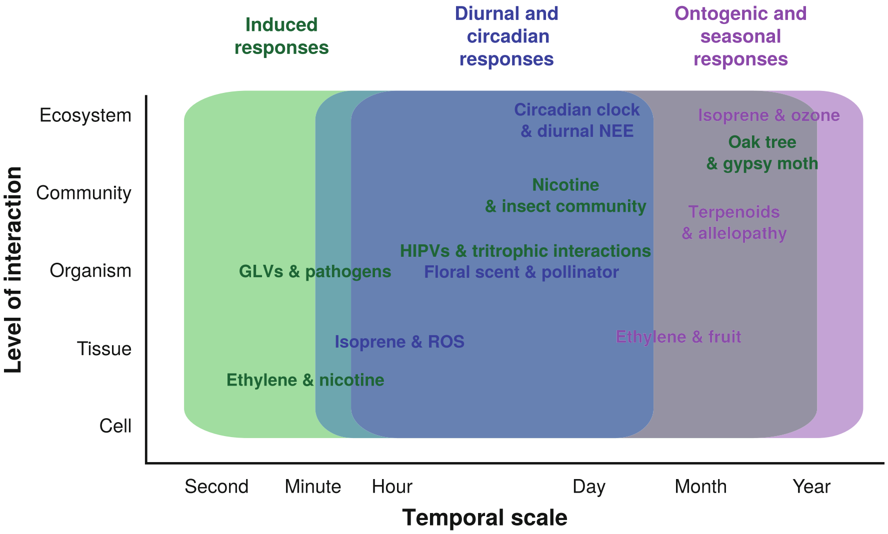

Fig. 1.5 A conceptual scheme of the functions of plant volatiles over ecological and temporal scales

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Chapter 2
Environmental Impacts on Plant Volatile Emission

Lucian Copolovici and Ülo Niinemets

Abstract Plants in their natural environment are often exposed to a variety of environmental stresses. This chapter emphasises the importance of distinguishing among stress effects on constitutive and stress-induced volatile emissions and, within constitutive emissions, among stress effects on emissions from specialised storage compartments (storage emissions) and de novo emissions. Among constitutive emissions, de novo emissions are typically more sensitive to stress than storage emissions. Depending on stress severity, the emission response is either physiological or the emission response is controlled at the gene expression level. This chapter analyses the impacts of heat, cold, drought and waterlogging stresses on constitutive and induced emissions, highlights similarities and differences of various stresses on volatile release and outlines the gaps in knowledge. We argue that for a fully mechanistic understanding of environmental impacts on plant chemical communication channels, more work is needed to obtain quantitative stress dose versus emission responses for different stresses in species of differing stress tolerance.

2.1 Introduction

Plants synthesise more than 100,000 chemical products and at least 1700 of these are known to be volatile. In the literature, the term *biogenic volatile organic compound* (BVOC) includes organic atmospheric trace gases synthesised by living

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organisms, typically excluding methane (Kesselmeier and Staudt 1999). Vegetation emits a wide array of different BVOC with isoprenoids (Fig. 2.1a), volatile fatty acid derivatives such as different C5 and C6 alcohols, aldehydes and ketones, and various benzenoids being quantitatively the most important compound classes (Arneth and Niinemets 2010; Fineschi et al. 2013; Niinemets et al. 2013).

The plant emissions can occur either under non-stressed conditions (constitutive emissions) or under stressed conditions (induced emissions and constitutive emissions) (Loreto and Schnitzler 2010; Niinemets 2010; Niinemets et al. 2010b). Only a limited number of plants are strong constitutive emitters of volatiles, but these emissions can often dominate the ecosystem, region and global emissions (Fineschi et al. 2013). Nevertheless, biotic and environmental stress can induce BVOC emissions in practically any plant species, and this can have a major impact on BVOC release from stressed ecosystems, with potential large-scale impacts (Arneth and Niinemets 2010; Grote et al. 2013). The induced emission of volatile organic compounds due to mild abiotic stress could prime the defence responses of plants upon exposure to a biotic stress (Conrath et al. 2006; Copolovici et al. 2014; Heil and Kost 2006; Niinemets 2010). On the other hand, plants that have been affected by a biotic stress could be protected from abiotic stresses due to enhanced elicitation of the chemical pathways responsible for the synthesis of protective compounds (Fujita et al. 2006) or due to the direct effect of these induced emissions on abiotic stress resistance (Owen and Peñuelas 2005). Furthermore, plants can signal neighbouring plants an imminent biotic attack using volatile organic compounds, and alteration of this signal by abiotic impacts could importantly affect the plant phenotypic response (Baldwin 2010; Baldwin et al. 2006), underscoring the importance of gaining an insight into the abiotic stress effects on BVOC emissions.

Among the constitutive emissions, volatile isoprenoids, including isoprene (5 carbon atoms, C5) and volatile terpenes such as monoterpenes consisting of two isoprene residues (C10) and sesquiterpenes consisting of three isoprene residues (C15; Fig. 2.1a), have traditionally been considered as the most important class of compounds released from plants due to the overall high contribution to total BVOC release and major roles in photosynthesis, respiration, membrane fluidity and biotic interactions (Fineschi et al. 2013; Harrison et al. 2013; Vranova et al. 2012). Furthermore, from a quantitative point of view, among the volatile isoprenoids, isoprene is globally the most important molecule released by plants into the atmosphere, followed by monoterpenes. The total isoprene emissions are estimated at about 550 Tg C year^{-1} and the total monoterpene emissions at about 100 Tg C year^{-1} (Arneth et al. 2008, 2010; Guenther et al. 2012). Due to their high reactivity, isoprene and terpenes play major roles in the determination of atmospheric reactivity and the formation of secondary organic aerosols and cloud condensation nuclei (Carlton et al. 2009; Engelhart et al. 2008; Hallquist et al. 2009; Kulmala et al. 2013).

Isoprenoids form a highly diverse class of compounds that are synthesised via two spatially separated pathways. The mevalonate (MVA) pathway is located in the cytoplasm and is used to synthesise volatile sesquiterpenes (Fig. 2.1) but also nonvolatile metabolites such as phytosterols (Gershenzon and Croteau 1993;

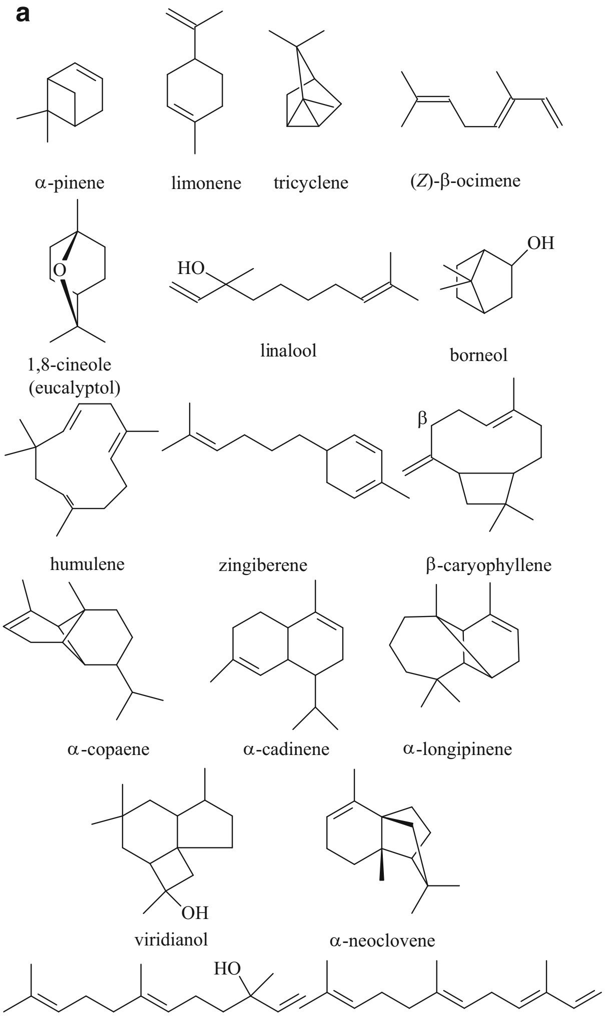

Fig. 2.1 Chemical structures of characteristic monoterpenes, sesquiterpenes and derivatives (**a**) and compartmentalisation of the biosynthetic pathways of volatile isoprenoids in plant cells (**b**). Typically, even single plant emissions consist of a complex blend of chemically heterogeneous volatiles. Often more than 20 different monoterpenes are emitted by a single species (Fineschi et al. 2013; Niinemets et al. 2004; Niinemets and Reichstein 2002). Monoterpenes can have acyclic or mono-, bi- and tricyclic structures. They are typically non-oxygenated with a few exceptions such as 1,8-cineole and linalool. There are a large number of oxygenated monoterpene derivatives. Sesquiterpenes can also be cyclic or acyclic, and there are many oxygenated derivatives. In (**b**), the C_{16} homoterpene (3*E*,7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) and C_{11} homoterpene (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) are also shown. As TMTT is synthesised from the diterpene geranyllinalool, its synthesis is expected to occur in the plastids as shown for the herbaceous legume *Lotus japonicus* (Brillada et al. 2013). However, in *Arabidopsis*, geranyllinalool and TMTT synthesis occur in the cytosol (Herde et al. 2008)

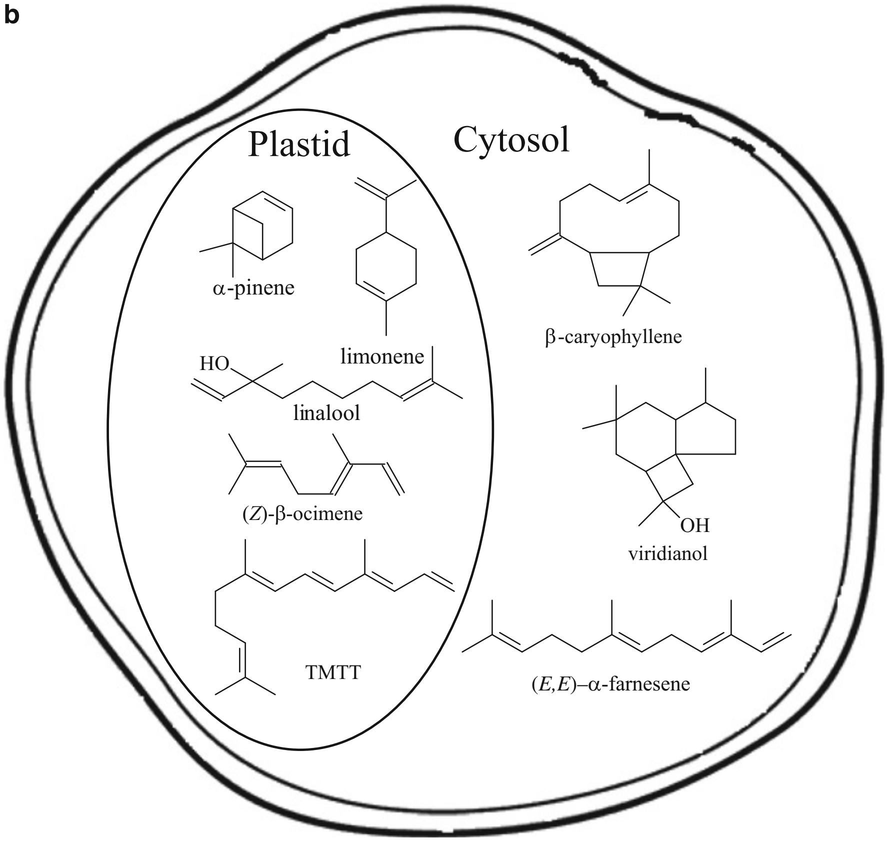

Fig. 2.1 (continued)

Lombard and Moreira 2011; Rosenkranz and Schnitzler 2013). The second pathway, the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, is located in plastids and is responsible for the synthesis of volatile isoprene and monoterpenes (Fig. 2.1) and multiple nonvolatile biologically highly relevant compounds such as carotenoids and the phytol residue of chlorophyll (Li and Sharkey 2013; Rajabi Memari et al. 2013; Rosenkranz and Schnitzler 2013). We refer to several recent reviews for details of pathway regulation and enzymatic synthesis of different isoprenoids (Baldwin 2010; Nakamura et al. 2001; Vranova et al. 2012).

Plants that are considered constitutive emitters of volatile isoprenoids can either be de novo emitters or storage emitters (Grote et al. 2013; Niinemets et al. 2010b). De novo emitters emit volatiles that have been immediately synthesised without being stored in a significant manner in plant tissues, while the emissions in storage emitters rely on compounds stored in specialised storage compartments and synthesised typically days to months prior to their release into the atmosphere. Constitutive de novo emitters mainly emit either isoprene (2-methyl-1,3-butadiene), 2-methyl-3-buten-2-ol (MBO) or monoterpenes, while storage emitters typically release mono- or sesquiterpenes or both (Azuma et al. 2010; Huang et al. 2012; Jardine et al. 2011; Kesselmeier and Staudt 1999; Staudt and Lhoutellier 2011). Several species have a mixed mode of emission relying both on de novo and storage emissions. In particular, many *Abies*, *Eucalyptus* and *Picea* species can emit simultaneously de novo synthesised isoprene and monoterpenes from storage (Harrison et al. 2001; Street et al. 1997; Westberg et al. 2000; Winters et al. 2009), while several *Pinus* species can emit de novo synthesised MBO and monoterpenes from storage (Gray et al. 2011; Harley et al. 1998).

In the case of de novo emitted compounds, the emission rate is controlled by temperature and light intensity that determine the pool size of precursors for compound synthesis (Li and Sharkey 2013; Rasulov et al. 2009, 2010), while the storage emissions are only dependent on temperature, which alters the diffusion flux out of the storage compartments (Grote et al. 2013; Guenther et al. 2012). Both types of emissions can be modified by compound physicochemical characteristics, and a mixed type of control is also possible (Niinemets et al. 2004; Niinemets and Reichstein 2002). Exposure to stress conditions can alter the rate of volatile emissions by different mechanisms. First, stress can change the rate of constitutive emissions, with the changes in the emission rate being either transient or sustained depending on the intensity and the duration of the stress. Second, stress can induce de novo synthesis of novel volatiles. Thus, stress can profoundly change the blend and overall emission rate of volatiles. Although there is evidence of convergence of early stress responses at the level of oxidative signalling (Fujita et al. 2006; Mittler 2006; Mittler et al. 2011) and there are several ubiquitous stress-dependent volatiles, different stresses can induce the release of different blends of volatiles and differentially affect constitutive and stress-dependent volatile emission rates. In this chapter, we analyse the effects of key abiotic stresses on plant volatile release, considering both the changes in the emission spectrum and the rate of emission. We argue that when analysing the effects of stress on emissions, it is important to clearly separate between the immediate physiological processes that modify the emission rate primarily due to changes in substrate pool sizes and gene expression level responses that lead to changes in the activity of key enzymes controlling the pathway flux and the composition of stress-dependent emissions.

2.2 Emission of Volatiles from Plants Under Abiotic Stresses

2.2.1 Stress-Elicited Volatiles

By definition, constitutively released volatiles are the compounds which are synthesised and released (or stored prior to their release in storage emitters) under normal physiological conditions, while stress-elicited volatiles (or induced volatiles) are produced de novo only in stress conditions. However, stress also elicits stress volatile release in constitutive emitters [reviewed by (Niinemets 2010)]. This can sometimes blur the separation of constitutive versus induced emissions. Specifically, in the case of constitutive terpene emitters, separation of terpenes released constitutively and upon exposure to stress might be difficult (Niinemets 2010; Niinemets et al. 2010a). Nevertheless, stress typically leads to a release of terpenes that are not emitted from unstressed plants. In particular, 1,8-cineole, linalool and ocimenes are characteristic stress-elicited monoterpenes (Aros et al. 2012; Konig et al. 1995). In addition, several stresses lead to the emission of the homoterpenes, (3*E*,7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT) and (3*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), and various sesquiterpenes, including β-caryophyllene, α-farnesene and Δ-elemene, although some of these sesquiterpenes can be emitted constitutively in some storage emitters (Blande et al. 2007; Joó et al. 2011; Niinemets et al. 2010b).

Apart from isoprenoids, many stress factors lead to major emissions of methanol as one of the first stress signals (Bamberger et al. 2010; Beauchamp et al. 2005; Filella et al. 2009). In non-stressed plants, especially in growing tissues, methanol is emitted as the result of demethylation of cell wall pectins by pectin methylesterases (Harley et al. 2007; Hüve et al. 2007). It is likely that stress-dependent emissions can be explained by the same mechanism (Peñuelas et al. 2005; Seco et al. 2011), although different pectin methylesterases can be activated upon stress (Pelloux et al. 2007).

Volatile lipoxygenase (LOX) pathway products, mainly consisting of various C6 alcohols and aldehydes (green leaf volatiles, GLV), constitute a ubiquitous class of compounds that are emitted in a variety of plant species during different stress conditions (Heiden et al. 2003; Kask et al. 2013; Niinemets 2010). They are typically emitted when the cellular membrane has been damaged (Kleist et al. 2012). The LOX pathway starts with a release of polyunsaturated fatty acids (octadecanoid acids) from plant membranes through the action of phospholipases (Liavonchanka and Feussner 2006). Further reaction with lipoxygenases produces 9- or 13-hydroperoxylinoleic or 9- or 13-linoleic acid or a mixture of both. Then, a hydroperoxide lyase catalyses the breakdown of 13-hydroperoxylinole(n)ic acid to a C6-compound, (*Z*)-3-hexenal, and a C12-product, 12-oxo-(*Z*)-9-dodecenoic acid. In consecutive reactions, (*Z*)-3-hexenal can be converted to (*Z*)-3-hexenol, (*E*)-2-hexenol, (*E*)-3-hexenol or (*E*)-2-hexenal (Feussner and Wasternack 2002; Matsui 2006). The release of GLV is typically considered de novo emission, although the substrate, polyunsaturated fatty acids, could have been synthesised and incorporated in membranes long before the stress event. In fact, recent experiments with ^{13}C-labelling indicated that the stress-dependent GLV emissions were not labelled by ^{13}C (Kleist et al. 2012)

In the following, we review the responses of key stress-elicited and constitutive emissions to high and low temperatures and to low and excess water availabilities. Although resolving the immediate and gene expression level effects of stress on different types of volatiles can be straightforward in several cases, processes with different time kinetics can overlap in others. Thus, stresses of different duration and severity can potentially lead to highly complex emission responses with potentially important consequences for communication of plants with other organisms and for plant-to-plant signalling.

2.2.2 High Temperature Impacts

Climate change involves rising temperature with a dramatic effect on human and natural systems. According to recent estimates, the global surface temperature has risen by about 0.8 °C over the past 150 years, and the temperature is predicted to keep increasing (Fyfe and Gillett 2014; Gillett et al. 2011). High temperatures constitute an important constraint for plants, restricting their productivity and growth and influencing the distribution of species (Loreau et al. 2001). In particular, photosynthesis is highly sensitive to heat stress due to inhibition of photosynthetic electron transport capacity and activity of Rubisco (Hüve et al. 2011; Niinemets and Keenan 2014; Parry et al. 2014; Way and Oren 2010). In addition to direct effects of heat on growth, exposure to heat stress can also critically alter the development of flowers and thereby modify the reproductive success and be therefore particularly important for the productivity of seed crops (Ainsworth and Ort 2010; De Storme and Geelen 2014). In fact, heat stress has a major impact on post-transcriptional and post-translational control systems, as well as on their targets, thereby affecting temperature stress tolerance (Guerra et al. 2015).

Several key plant volatiles have been shown to improve heat stress resistance and have consequently been at the centre of research into heat resistance, although the exact mechanisms of their action are not fully understood (Vickers et al. 2009). In addition to compounds likely involved directly in heat resistance, multiple other compounds are released as the result of activation of ubiquitous stress pathways.

2.2.2.1 Isoprene Emission

In constitutively isoprene-emitting species, even a mild heat stress can enhance isoprene emission due to transient upregulation of the isoprenoid synthesis pathway as the result of greater substrate pools for isoprene synthesis and greater activity of isoprene synthases (e.g. Niinemets 2010; Rasulov et al. 2010). In addition to the transient enhancement of emissions, longer-term increase of temperature leads to acclimation responses typically associated with enhancement of expression of the isoprene synthase gene as well as genes controlling the entire plastidic isoprenoid synthesis pathway (Hanson and Sharkey 2001; Wiberley et al. 2008), but also with leaf structural changes altering the amount of physiologically active, isoprene-producing, leaf biomass per unit leaf area (Rasulov et al. 2015). For example, the rate of isoprene emission was greater from seedlings of the isoprene-emitting deciduous tree species *Populus nigra* (Centritto et al. 2011) and *Populus tremula* × *P. tremuloides* (Rasulov et al. 2015) grown at 35 °C than from seedlings grown at 25 °C. Analogously, in the grass *Phragmites australis*, an increase of soil temperature also led to increases in isoprene emission rate (Medori et al. 2012). However, increases in night-time temperature did not affect significantly isoprene emission in seedlings of *Populus tremula* (Ibrahim et al. 2010), suggesting that it is not the overall increase in daily temperature but primarily the increase in temperature during the photosynthetic period.

Sharkey and Singsaas (1995) have demonstrated that isoprene can protect photosynthetic apparatus against damage caused by transient high temperature stress, and their finding has been supported by several further studies (Pollastri et al. 2014; Sharkey et al. 2008; Sun et al. 2013; Velikova et al. 2006; Wang et al. 2014). Furthermore, these results were supported by studies with genetically engineered grey poplar (*Populus x canescens*) lacking isoprene emission due to silencing of the isoprene synthase gene (Behnke et al. 2007, 2013). These researchers demonstrated that non-isoprene-emitting plants were more sensitive to recurrent heat stress simulating heat flecks that can occur upon sudden exposure to full sunlight (Behnke et al. 2007). Heat flecks frequently occur under dynamic light conditions in the field (Sharkey et al. 1996; Singsaas and Sharkey 1998), and in fact, isoprene as a small highly volatile molecule might confer the greatest protection under such dynamic conditions rather than under sustained high temperature conditions (Fineschi et al. 2013; Niinemets and Sun 2015; Sun et al. 2013).

2.2.2.2 Terpene Emissions

Constitutive terpene emissions from storage pools usually increase with increasing temperature, reflecting the exponential temperature dependency of diffusion and terpene vapour pressure (Grote et al. 2013; Guenther et al. 1993). Analogously to isoprene emissions, immediate effects of high temperatures on terpene emissions by de novo emitting species can result from enhanced precursor pool sizes and enhanced terpene synthase activities. Indeed, de novo synthesis of volatiles often increases due to temperature effects on the enzymes responsible for volatile synthesis (Grote et al. 2013; Niinemets et al. 2010b). Emission of de novo synthesised monoterpenes has been shown to improve foliage heat resistance (Copolovici et al. 2005; Llusià et al. 2005; Loreto et al. 1998), suggesting that monoterpenes operate similarly to isoprene, although different monoterpenes have differing capacities for heat protection (Copolovici et al. 2005). However, in contrast to storage emissions, the release of de novo synthesised monoterpenes has a temperature optimum that is generally lower, at around 35 °C (Copolovici et al. 2005; Loreto et al. 1998), than the temperature optimum for isoprene synthesis of around 40–45 °C (Rasulov et al. 2010, 2015). Nevertheless, less volatile monoterpenes can be nonspecifically stored in leaf liquid and lipid phases (Niinemets and Reichstein 2002; Niinemets et al. 2002) and, thus, could protect leaves for longer periods than isoprene. In addition, due to maintenance of significant concentrations in leaf tissues, they can still be involved in protection from heat flecks under dynamic light conditions at temperatures exceeding the optimum temperature for their synthesis.

Apart from the effects on constitutive emission rates, heat stress elicits expression of multiple terpenoid synthase genes (Mazzucotelli et al. 2008). In the conifer *Pseudotsuga menziesii*, heat stress-enhanced emissions of sesquiterpenes, the monoterpene alcohol linalool and the benzenoid methyl salicylate and the increases were much less for other monoterpenes (Joo et al. 2011). Analogously, heat stress led to emission of sesquiterpenes and selective effects on different monoterpenes in the herb *Solanum lycopersicum* (Fig. 2.2, Copolovici et al. 2012). These changes in the emission composition have been associated with altered gene expression profiles. However, alterations in emission composition by heat stress in *P. menziesii*, where terpenoids are stored in resin ducts, and in *S. lycopersicum*, where terpenoids are stored in glandular trichomes, can partly reflect stronger increases in the volatility of C15 relative to C10 compounds as well as effects of temperature on the permeability of cuticle and cellular structures. On the other hand, in the deciduous broad-leaved tree *Quercus rubra*, a constitutive isoprene emitter, heat stress resulted in elicitation of de novo monoterpene emissions that increased after

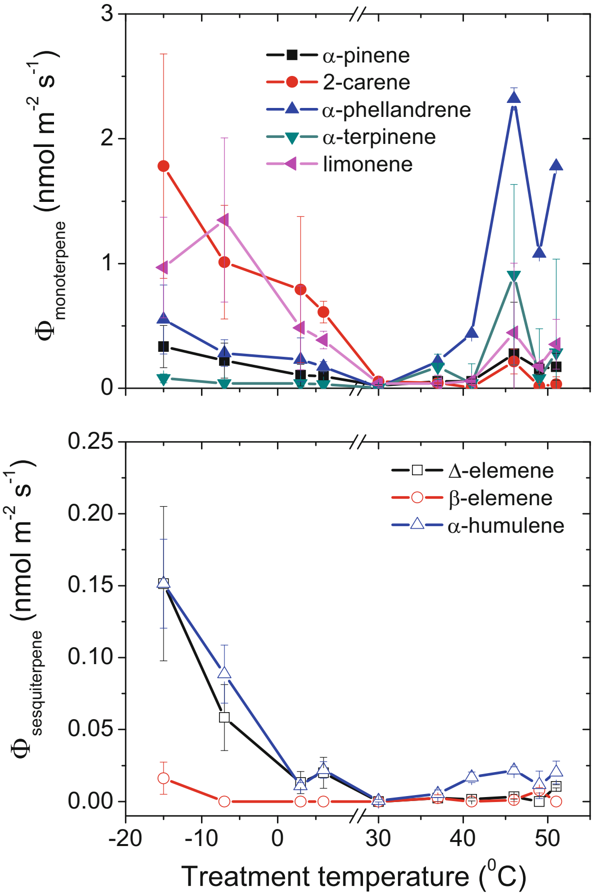

Fig. 2.2 Illustration of the effects of high and low temperatures on the release of mono- and sesquiterpenes from the foliage of the herb *Solanum lycopersicum* (modified from Copolovici et al. 2012)

the first hour of mild stress (45 °C) and were maintained at the induced level for the rest of the treatment (Copolovici et al. unpublished data). In a similar manner, in the evergreen broad-leaved *Quercus ilex*, a constitutive de novo monoterpene emitter, heat stress led to synthesis of ocimenes, linalool and sesquiterpenes (Staudt and Bertin 1998).

2.2.2.3 Release of Green Leaf Volatiles

As discussed in Sect. 2.1, green leaf volatiles (GLV) constitute a ubiquitous class of compounds released in response to virtually any stress (for a review, see Niinemets 2010). Given that the release of GLV is associated with membrane-level damage, it is expected that GLV is emitted upon severe heat stress. However, a certain elevation of GLV emission can already occur upon a moderate increase in temperature, e.g. in *Betula pendula* (Hartikainen et al. 2012) and *Picea abies* (Filella et al. 2007). In *S. lycopersicum* (Copolovici et al. 2012) and in a number of tree species (Kleist et al. 2012), it has been further demonstrated that the emission rate of GLV can be correlated with the stress strength, indicating that GLV release can serve to quantify the severity of damage upon heat exposure. Overall, it is expected that future heat waves will result in enhanced release of constitutive and induced terpenoids and GLV emissions.

2.2.3 Low Temperature Impacts

The yield potential and geographic range of crops is importantly modulated by low temperatures (Harley 2011), especially by early and late season frosts (Kalisz et al. 2014; Kim et al. 2013; Luo et al. 2014). Studies have mainly investigated the responses of photosynthesis to chilling and frost stress (Adams et al. 2001; Küppers and Küppers 1999; Savitch et al. 2002), and there is much less information available of low-temperature effects on the emission of volatile organic compounds.

Cold stress in *Populus tremula* led to reductions in constitutive leaf isoprene emissions, but after the cold stress, isoprene emissions partly recovered due to increases in the pool size of the immediate isoprene precursor and the fraction of carbon going into isoprene synthesis (Sun et al. 2012a). Induction of the release of certain stress-dependent mono- and sesquiterpenes has been observed after a cold shock treatment in *S. lycopersicum* (Copolovici et al. 2012). In this species, the emission of (*E*)-β-ocimene was quantitatively correlated with the severity of temperature stress, with emissions at levels higher than 1 pmol m^{-2} s^{-1} only observed after severe stress (temperatures <1 °C) (Copolovici et al. 2012). The emission of sesquiterpenes, especially β-caryophyllene, after cold shock treatment was quantitatively correlated with stress strength during the treatment (Copolovici et al. 2012). Both induction of terpenoid synthesis after stress and breakage of primarily sesquiterpene-containing structures could have played a role in enhanced sesquiterpene release in this study. In fact, Copolovici et al. (2012) demonstrated that exposure of *S. lycopersicum* leaves to freezing temperatures led to the release of GLV including (*Z*)-3-hexenol, (*E*)-2-hexenal, 1-hexanol and 1,4-hexadienal, whereas the emissions increased drastically below a temperature threshold. Cold shock treatments in the deciduous vine *Vitis vinifera* resulted in a similar enhancement of GLV production as well as enhanced production of nonvolatile GLV condensation products with glutathione (S-(3-hexan-1-ol)-glutathione) and cysteine (S-(3-hexan-l-ol)-ʟ-cysteine) (Kobayashi et al. 2011). These nonvolatiles of *V. vinifera* are known precursors of S-containing aroma compounds such as 3-mercaptohexan-1-ol (Peyrot Des Gachons et al. 2002), implying that cold shock can have major impacts on species-specific smell bouquets.

2.2.4 Influence of Drought on Plant Emissions

Temporal or chronic drought is a major stress factor worldwide. According to the Food and Agriculture Organization (FAO) of the United Nations, chronic limitations of water availability affect at least 40 % of the Earth's surface (Bodner et al. 2015; FAO 2003). The negative impact of drought on photosynthesis, due to reduced CO_2 entry into chloroplasts as the result of decreases in stomatal and mesophyll diffusion conductances, and reductions in Rubisco activity and photosynthetic electron transport rate are well-known (Flexas et al. 2014, 2016; Galmés et al. 2012; Niinemets and Keenan 2014). However, as demonstrated in the following sections, the effects of drought on constitutive isoprenoid emission are somewhat controversial.

2.2.4.1 Effects of Drought on Isoprene Emission

In the case of constitutive isoprene emissions, there might seem to be no clear trend across studies if one does not consider that the effects of drought depend on species, stress intensity and timing (Niinemets et al. 2010a; Peñuelas and Staudt 2010). Peñuelas and Staudt (2010) summarised the available evidence and indicated that in about 25 % of studies, isoprene emission rates increased; in about 50 % of studies, the emissions decreased; and in about 25 % of studies, the emission rate was not changed.

Indeed, the response of constitutive isoprene emissions to drought includes both direct physiological responses as the result of drought effects on CO_2 concentration in chloroplasts and acclimation responses as the result of changes in isoprene synthase gene expression. Regarding the physiological response, isoprene emission depends on ambient CO_2 concentration according to a curve with an optimum at relatively low intercellular CO_2 concentrations of 100–150 μmol mol^{-1} (Rasulov et al. 2009; Sun et al. 2012b; Wilkinson et al. 2009). Thus, mild water stress that leads to moderate reductions in stomatal conductance can lead to increases in isoprene emission, as demonstrated in several studies (Funk et al. 2004; Genard-Zielinski et al. 2014; Niinemets et al. 2010a; Pegoraro et al. 2004; Sharkey and Loreto 1993). Moderate drought during the middle of the summer can even cause a doubling of isoprene emission compared with well-watered periods (Monson et al. 2007). With further advancement of drought, isoprene emission rate typically decreases (Fang et al. 1996; Sharkey and Loreto 1993). When this happens, the recovery upon watering can be time-consuming, taking 4 days or more, and indicating that drought has led to a reduction of isoprene synthase activity such that recovery critically depends on changes in expression of isoprene synthase activity. On the other hand, recovery can result in overcompensation such that the emissions after stress are higher than before the stress (Niinemets 2010, 2016; Sharkey and Loreto 1993).

The direct impacts of drought-dependent changes in isoprene emission are not clear. Experiments with isoprene-emitting transgenic *Nicotiana tabacum* plants demonstrated that drought-stressed non-isoprene-emitting plants had increased foliar concentrations of reactive oxygen species (ROS) and greater lipid peroxidation level, while no change in ROS and lipid peroxidation occurred in isoprene-emitting plants (Ryan et al. 2014). Thus, increases in isoprene emission upon moderate drought stress could play a role in maintenance of membrane integrity in drought-stressed plants.

2.2.4.2 Drought Influences on Terpene Emissions

Similar to constitutive isoprene emissions, effects of drought on monoterpene emissions are variable. In the constitutive de novo monoterpene emitters *Quercus ilex* and *Fagus sylvatica*, drought enhanced the emission rate (Blanch et al. 2009a; Wu et al. 2015). In contrast, drought-dependent reductions in monoterpene emission have been observed in other studies with *Q. ilex* (Lavoir et al. 2009; Plaza et al. 2005; Staudt et al. 2002). The study of Wu et al. (2015) indicates that this discrepancy likely reflects differences in the severity of drought. In their study, monoterpene emission in both *Q. ilex* and *F. sylvatica* was initially enhanced and then severely curbed as the drought period continued (Wu et al. 2015). We suggest that the initial increase of monoterpene emissions reflects the effects of low intercellular CO_2 concentrations on the substrate pool size similar to isoprene (see above), while the severe stress leads to reductions in monoterpene synthase activity, although the latter effect has not always been found (Grote et al. 2010). Similar to isoprene, overcompensation of monoterpene emissions upon rewatering has sometimes been observed (Peñuelas et al. 2009).

In the case of the constitutive storage monoterpene emitters *Cistus albidus*, *Pinus halepensis*, and *Rosmarinus officinalis* drought stressed for 11 days, drought initially had a minor effect on monoterpene emissions, but there was a surprising enhancement at day 7, followed by a reduction during the remaining days of the experiment, whereas the emissions still remained higher at the end of the experiment than the emissions in non-stressed plants (Ormeño et al. 2007). In contrast, storage emissions in *Picea abies* and *Pinus sylvestris* were weakly affected by drought (Wu et al. 2015). The drought-dependent increase of monoterpene emissions observed in some storage emitters has been suggested to indicate greater investment in secondary carbon-rich chemicals, including terpenes, in conditions that lead to an arrestment of primary metabolism and growth (Blanch et al. 2009a; Delfine et al. 2005). Indeed, increases in monoterpene content upon a moderate water stress have been observed in *R. officinalis* and *Mentha spicata* (Delfine et al. 2005), in *P. halepensis* (Blanch et al. 2009b) and in two *Eucalyptus* species (McKiernan et al. 2014). However, upon a severe stress, the investment of carbon into secondary chemistry also declines and the storage pools for terpenes decrease, leading to reduced terpene content as has been observed, for example, in the conifer *Cupressus sempervirens* (Yani et al. 1993).

Much less data are available for drought effects on constitutive sesquiterpene and stress-induced volatile emissions. In the storage emitter *R. officinalis*, sesquiterpene emissions decreased drastically under drought stress, but minor effects were observed for *C. albidus* and *P. halepensis* (Ormeño et al. 2007). However, it is unclear as to what extent sesquiterpene emissions in their study reflected emissions from storage or induction of emissions. In the de novo emitter *Q. coccifera*, sesquiterpene emissions decreased with advancing drought, but there was a certain increase at the end of the experiment under most severe water stress conditions (Ormeño et al. 2007). In wheat (*Triticum aestivum*) plants, sustained drought was associated with the release of monoterpenes, benzaldehyde and geranyl acetone, and these emissions were smaller in bacterially primed plants (i.e. inoculated with biofilm-forming bacteria) that were more resistant to drought (Timmusk et al. 2014). Only a moderate drought effect was observed on induced mono- and sesquiterpene emissions in the broad-leaved deciduous species *Quercus robur* and *Prunus serotina* (Bourtsoukidis et al. 2014). In the latter study, methanol emissions were reduced and GLV emissions were either reduced or unchanged upon drought exposure (Bourtsoukidis et al. 2014). In contrast, in broad-leaved deciduous species *Betula pendula*, drought enhanced both GLV and monoterpene emissions (Pag et al. 2013). There is evidence that drought can enhance methyl salicylate emissions (Bourtsoukidis et al. 2014; Copolovici et al. 2014), and there is further evidence that drought might prime volatile defences for a stronger response upon biotic attack (Copolovici et al. 2014). We conclude that more experimental work is needed on drought effects on induction of isoprenoids and on emission of immediate stress volatiles.

2.2.5 Influence of Flooding on Plant Emissions

The effects of flooding on different plant species depend on plant waterlogging tolerance and on the intensity of stress, stress timing and site conditions (Kozlowski and Pallardy 2002). Some species can only tolerate short episodes of flooding, while others can grow in permanently flooded soils. Anoxic conditions elicit a plethora of physiological stress responses including reductions in photosynthesis rates and stomatal conductance (Jackson et al. 2009). The key adaptations to cope with flooding include modifications that improve oxygen availability such as root regeneration, facilitation of oxygen uptake and transport and metabolic adjustments (Bertolde et al. 2012; de Oliveira et al. 2014; Yu et al. 2015).

Although flooding is a major stress in several ecosystems, flooding effects on BVOC emissions have received little coverage in the literature (Kreuzwieser and Rennenberg 2013), except for the classical increases of emissions of ethanol and acetaldehyde from foliage of waterlogged plants (Holzinger et al. 2005; Kreuzwieser et al. 1999, 2000, 2001) that arise as the result of anaerobic metabolism of sugars in the roots. In addition to major emissions of ethanol and acetaldehyde, flooding elicits a release of a series of volatile stress marker compounds including methanol (Bourtsoukidis et al. 2014; Copolovici and Niinemets 2010; Rottenberger et al. 2008), and GLV (Fig. 2.3) (Bourtsoukidis et al. 2014; Copolovici and Niinemets 2010). Apart from the C6 GLV, Bourtsoukidis et al. (2014) observed significant emissions of the C9 oxylipin nonanal—an indicator of degradation of lipid membranes—in *Quercus robur* and *Prunus serotina*. In addition to carbon-based volatiles, Copolovici and Niinemets (2010) observed significant emissions of NO upon flooding in three deciduous species: *Alnus glutinosa*, *Populus tremula* and *Quercus rubra*. The rate of NO emissions correlated with the rate of GLV release, and the rate of emissions of both NO and GLV increased with decreasing species flooding tolerance (Copolovici and Niinemets 2010).

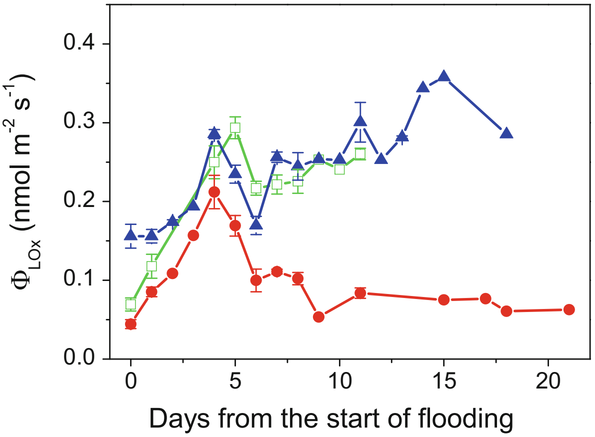

Fig. 2.3 Time-dependent changes in lipoxygenase (LOX) pathway products (green leaf volatiles) in three temperate deciduous tree species *Alnus glutinosa* (*red line* and *symbols*), *Populus tremula* (*green line* and *symbols*) and *Quercus rubra* (*blue line* and *symbols*) after the onset of a waterlogging treatment. *Alnus glutinosa* is the most and *Q. rubra* the least waterlogging-tolerant species (Niinemets and Valladares 2006). Data are modified from Copolovici and Niinemets (2010)

In general, flooding results in reductions in constitutive isoprene emission as the data for *Q. rubra* and several Amazonian species (Bracho Nunez et al. 2009) demonstrate. However, in *P. tremula* that is more resistant to waterlogging than *Q. rubra*, isoprene emission was marginally affected and even fully recovered during the flooding treatment (Copolovici and Niinemets 2010). Surprisingly, no effect of flooding was evident on isoprene emissions in a relatively flooding-intolerant species *Q. robur* (Bourtsoukidis et al. 2014). Given that flooding results in simultaneous reductions in both stomatal conductance and net assimilation rate such that intercellular CO_2 concentration remains essentially unaltered (Copolovici and Niinemets 2010), no increase of isoprene emissions even under mild stress are expected, and the time-dependent reduction of isoprene emission rate under sustained flooding likely reflects decreases in isoprene synthase activity.

Few data are available for constitutive and induced terpene emissions. In several constitutive monoterpene-emitting Amazonian species (Bracho Nunez et al. 2009), flooding reduced emissions. In contrast, for induced monoterpenes, the flooding effect was not significant, but there was a moderate increase in induced sesquiterpene emissions in two temperate deciduous species (Bourtsoukidis et al. 2014). More studies on the effects of flooding on constitutive isoprene and monoterpene emitters are needed to gain conclusive insight into the species and stress severity controls on emissions.

Different to other stresses, the release of NO from flooded plants can lead to the interesting possibility of altered air reactivity without anthropogenic pollution. Both reactive hydrocarbons that are primarily biogenic, and NO_X (NO and NO_2) that are primarily anthropogenic are needed for ozone formation in the troposphere (Fall 2003). Thus, sustained high-level NO emissions from flooded isoprene-emitting *Q. rubra* trees (Copolovici and Niinemets 2010) suggest that ecosystems dominated by this species may be significant producers of ozone in the absence of human-driven NO_X production. Although flooding significantly reduced isoprene emissions in *Q. rubra*, the emissions still remained at a level of 30–50 % after sustained flooding (Copolovici and Niinemets 2010), implying that flooded forest ecosystems keep altering air quality. In addition, oxygenated compounds, ethanol, acetaldehyde, GLV and methanol are emitted from flooded plants (Bourtsoukidis et al. 2014; Bracho Nunez et al. 2009; Copolovici and Niinemets 2010). Although the reactivity of these compounds is lower than that of non-oxygenated non-saturated hydrocarbons (Jiménez et al. 2007), they still significantly contribute to atmospheric OH radical and O_3 formation. Quantitative relationships between flooding tolerance, and time of flooding are needed to predict emissions of NO and oxygenated and non-oxygenated BVOC from flooded forests.

2.3 Conclusions and Suggestions for Future Work

This chapter demonstrates that a variety of emission responses are observed from stressed plants. Although all environmental stresses bear similarities, e.g. any stress typically leads to reductions in leaf photosynthesis rates, different stresses differently affect volatile emission rates, and the responses can be different for constitutive and induced emissions. In addition, for any stress, the effects depend on stress severity and duration. Mild stress characteristically first results in physiological responses that are quickly reversible upon a return to non-stressed conditions. Such physiological responses typically result from changes in substrate availability for all stresses and from changes in enzyme activity for temperature stresses. Thus, the effects can be positive, e.g. due to enhanced substrate availability for isoprene emission upon mild drought stress or due to enhanced substrate availability and enzyme activity upon mild heat stress. For other mild stresses, the effects can be negative or occasionally no effects can be observed. Mild stress seldom elicits release of stress volatiles, or if it does, the elicitation is minor. More severe stress typically leads to major reductions in constitutive emissions and release of characteristic stress volatiles. The available evidence demonstrates that the release of stress volatiles is stress dose dependent.

This chapter indicates the existence of important gaps in understanding the mechanisms of action of several stress factors and also indicates that there is a limited coverage of stress effects on emissions of several compound classes. The major limitation in a number of past studies has been that stress severity has not been objectively assessed, making it difficult to judge whether a certain phenomenon observed in a given study reflects a physiological response or a severe stress response. We argue that more experimental work is needed to fill the gaps in knowledge on stress responses of volatile compound classes with limited measurements, such as sesquiterpenes. We also suggest that for fully mechanistic consideration of environmental stress effects on BVOC emissions, stress dose versus emission relationships need to be developed using quantitative approaches to characterise the severity of stress.

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Chapter 3
Impacts of Induction of Plant Volatiles by Individual and Multiple Stresses Across Trophic Levels

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Abstract Plants are constantly challenged by many different stresses, ranging from abiotic factors, such as ultraviolet light and ozone, to herbivores and pathogens. To defend themselves against these challenges, plants activate defences that are specific to each stressor. One such defence is the emission of induced volatile organic compounds (VOCs) that can directly reduce the intensity of the stress or, in the case of herbivores, attract predators and parasitoids, in what is known as indirect defence. In nature, however, plants are rarely subject to stress by a single agent. In this chapter, we review what is known about the ecological effects of induced plant VOCs against individual and multiple stresses. First, we describe the biochemical responses against individual stressors that result in the emission of VOCs and how they can be modified by multiple stresses. We then discuss how plant VOCs can have an impact on herbivores, herbivore natural enemies and plant mutualists. We finish by discussing how future research should begin to investigate the importance of induced responses to multiple stresses in structuring plant-based communities.

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3.1 Introduction

Plants provide the template for most of the interactions observed in terrestrial ecosystems. The way they respond to different stresses can create spatially and temporally dynamic patterns of plant phenotypes that can affect the function of entire ecosystems. On a local scale, short-term induced plant responses can influence herbivore colonisation, performance and mortality. On larger scales, variation in community structure can lead to the evolution of different defensive responses in different populations, creating a shifting mosaic of co-evolutionary interactions (Thompson 2005; Berenbaum and Zangerl 2006). Plants are often more permanent members of the community than many of their herbivores. Thus, through defensive responses that persist through time, plants can link organisms that are separated temporally. In addition, they link above- and belowground compartments in ecosystems through plant-wide, systemic phenotypic changes resulting from responses to stress. Finally, decaying plant material can carry the legacy of plant defence responses and have an impact on decomposers and nutrient cycling.

Plant mediation of community structure is critically dependent on how plants respond to stress caused by biotic and abiotic factors. Upon stress, plants emit a novel blend of volatile organic compounds (VOCs) with more than one potential ecophysiological role. In plant–arthropod interactions, VOCs drive the search for suitable oviposition sites (Bruce et al. 2005) and food sources by herbivores and higher trophic levels (Schoonhoven et al. 2005; Dudareva et al. 2006), and they guarantee the reproductive success of plants by recruiting pollinators (Raguso 2004). Induced VOCs are known to attract or repel other herbivores (De Moraes et al. 2001; Zakir et al. 2013), recruit predators and parasitoids (Heil 2008) and mediate plant–plant communication (Heil and Ton 2008). The stresses that induce responses in plants, including VOC emission, range from herbivore feeding and oviposition, pathogen attack to abiotic stressors such as ultraviolet (UV-) light and ozone (Loreto and Schnitzler 2010). Induction of plant defence pathways can also occur in response to mutualists such as endophytes and mycorrhiza (Hartley and Gange 2009; Saikkonen et al. 2013). Thus, plant VOCs constitute a complex web of potential information that is superimposed on the food web (Dicke 2006).

We have accumulated substantial information on the mechanisms of induction of VOCs in response to individual stressors, and how these may impact simplified plant–herbivore–natural enemy systems. However, central to our understanding of these ecological effects is how plants deal with multiple stresses that occur either simultaneously or sequentially. Considering the plethora of organisms that interact with the plant and the variable abiotic environment that surrounds it, this scenario of multiple stress is the rule rather than the exception in nature. We are only beginning to uncover how these mechanisms interact when a plant is subjected to multiple stress factors simultaneously, and how these effects can percolate through more complex communities. Plant-mediated interactions are increasingly seen as fundamental parts of agroecosystems (Inbar and Gerling 2008; Braasch et al. 2012;

Kaplan 2012), so understanding plant responses to stress and their effects on other organisms will be increasingly important for enhancing sustainable food production.

3.2 Induction of VOCs by Biotic and Abiotic Stress

3.2.1 VOCs as a Common Induced Response to Stress

When subjected to stress, plants respond with an array of metabolic changes that result in the expression of defensive traits, thus modifying the plant's defensive phenotype. These changes include the induction of physical barriers as well as increases in the concentration of secondary metabolites, including plant VOCs. The induction of these plant responses to stress is a result of the activation of orchestrated signal transduction pathways triggered by recognition of the stressor. In the case of biotic stress, herbivore- or pathogen-derived elicitors (Maffei et al. 2012) trigger a series of biochemical events and the accumulation of signalling molecules that result in the induction of localised and systemic plant responses (for reviews, see Arimura et al. 2009; Wu and Baldwin 2009; Arimura et al. 2011) like the induction of VOCs. Some signals that trigger these responses to herbivory, such as the accumulation of reactive oxygen species (ROS), are common to various stresses (Apel and Hirt 2004; Petrov et al. 2015) and probably a convergence point between the signalling pathways activated in the plant (Fujita et al. 2006).

Plant responses to stress are modulated mainly by activation of three signalling pathways, regulated by the hormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) (Bari and Jones 2009). Production of these signalling molecules varies in quantity, composition and timing, giving the response to a specific stress its own fingerprint (Koornneef and Pieterse 2008). In plant–herbivore interactions, JA-dependent gene expression is involved in the majority of plant responses to chewing herbivores. In contrast, SA-dependent responses are activated upon feeding by sucking insects such as whiteflies and aphids that have a long-lasting and intimate relationship with the plant cell. These responses resemble those against pathogens that also activate the SA-signalling pathway (Walling 2000; Yuan and Lin 2008). Therefore, plant defence responses to pathogens, herbivores and abiotic stress can often overlap (Thaler et al. 2010).

The induction of VOCs upon herbivory has been extensively studied, perhaps because of its well-known role in mediating indirect plant defence (Heil 2008) and its potential to enhance biological control in agricultural crops. Plants can also produce and emit VOCs in response to pathogen infection (Cardoza et al. 2002; Rostás et al. 2006) as well as abiotic factors including high temperatures, light intensity (Loreto et al. 2006), elevated ozone (Vuorinen et al. 2004; Cui et al. 2014) and water and salt stress (Loreto and Delfine 2000; Teuber et al. 2008; Holopainen and Gershenzon 2010). The induction of VOCs upon abiotic stress has been proposed as a defence mechanism, as it can mitigate damage to the plant's machinery caused by extreme conditions (Holopainen and Gershenzon 2010; Loreto and Schnitzler 2010). Monoterpenes, for instance, can quench ozone preventing membrane oxidation (Loreto et al. 2004; Vickers et al. 2009) and have been implicated in increased thermotolerance (Loreto et al. 1998; Velikova and Loreto 2005).

Different stressors induce VOC bouquets that are either qualitatively or quantitatively unique (for example Cardoza et al. 2002; Vuorinen et al. 2004). These novel blends, however, share well-known semiochemical compounds belonging to the group of fatty acid derivates (green-leaf volatiles -GLVs), terpenoids and phenylpropanoids. For instance, methyl salicylate (MeSA), induced by mites (Ament et al. 2004) and aphids in many different systems (Glinwood and Pettersson 2000; Pareja et al. 2009; Salamanca et al. 2015), is also induced in tobacco plants upon acute ozone exposure (Heiden et al. 1999) as well as in peanut plants upon pathogen infection (Cardoza et al. 2002). Therefore, the induction of VOCs by stress other than herbivory is relevant in natural conditions.

Plant biochemical responses to multiple stresses are complicated by interactions between different signalling pathways. Induction of different pathways can synergise or inhibit each other. In particular, evidence suggests that JA and SA signalling can interact negatively, so induction of one pathway generally downregulates gene expression induced by the other (Bostock 2005; Mittler 2006; Koornneef and Pieterse 2008; Thaler et al. 2012). This interplay, often called signal crosstalk, means that induction of multiple pathways can result in highly specific stress responses that depend on feeding mode, characteristics of herbivore/pathogen molecular patterns as well as stress intensity, duration and timing (Thaler et al. 2004; Holopainen and Gershenzon 2010; Loreto and Schnitzler 2010). This, in turn, has important ecological consequences for two reasons. First, herbivores can manipulate this crosstalk to their own benefit, switching off effective defences. Second, the extent of crosstalk between pathways has an important influence on how multiple damage events affect the community of organisms associated with a particular plant.

3.2.2 Herbivore Manipulation of Plant Responses

Plant responses have long been assumed to be adaptive and defensive, though there is so far little evidence that induced VOC emission has been under selective pressure (Peñuelas and Llusià 2004). Furthermore, it is becoming clear that, in some cases, induced responses can be detrimental to the plant, since herbivores can manipulate plant responses to overcome effective defences and improve their performance. This manipulation of plant defences has been shown for arthropods belonging to different taxonomic groups with different feeding habits such as phloem feeders (Zarate et al. 2007; Walling 2008; Bos et al. 2010), caterpillars (Musser et al. 2002), beetles (Chung et al. 2013) and spider mites (Sarmento et al. 2011) and proposed as a mechanism exploited by herbivores to widen host plant range (Eichenseer et al. 2010). The biochemistry of this manipulation is mainly the result of SA-mediated inhibition of JA biosynthesis and downregulation of JA-dependent genes in negative crosstalk between signalling pathways (Zarate et al. 2007; Diezel et al. 2009), though it should be pointed out that SA-independent suppression of JA-induced plant defences has also been found (Musser et al. 2005; Weech et al. 2008; Sarmento et al. 2011). Defence manipulation is driven by effectors present in the saliva of herbivores such as the enzyme glucose oxidase (GOX) in caterpillars (Musser et al. 2002; Eichenseer et al. 2010), which suppresses wound-induced anti-herbivore defences (Musser et al. 2005; Diezel et al. 2009). GOX is known to manipulate terpenoid biosynthesis by suppressing transcripts encoding enzymes involved in the biosynthetic pathway (Bede et al. 2006), and GOX-mediated suppression of defences can improve the performance of neonate caterpillars (Musser et al. 2002). Likewise phloem feeders (whiteflies and aphids) can suppress JA-dependent induced plant defences and improve performance via effectors present in the saliva that are injected into the plant (Zarate et al. 2007; Walling 2008; Bos et al. 2010). Herbivores can also rely on associations with microorganisms to downregulate plant defences. For example at least one bacterium in the oral secretions of the Colorado potato beetle *Leptinotarsa decemlineata* is responsible for suppressing plant defences and enhancing larval growth of neonate larvae on tomato plants (Chung et al. 2013).

Because induction and suppression of plant defences involve several interconnected biochemical pathways, under natural conditions herbivore-induced VOCs may be the result of elicitors and suppressors of plant defences present in oral secretions. Feeding by the mite *Tetranychus evansi*, for example, downregulates expression of the terpene synthase GGPS1 in tomato plants (Sarmento et al. 2011), which produces the precursor of (*E*,*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT), a *Tetranychus urticae*-induced homoterpene involved in the recruitment of predatory mites (de Boer and Dicke 2004). There is evidence that feeding by *Heliothis virescens* caterpillars with impaired salivary glands results in the emission of a qualitatively and quantitatively different VOC blend in *Nicotiana tabacum* compared with that induced by intact caterpillars, suggesting that effectors present in the saliva suppress volatile compounds that include several terpenoids and nicotine (Delphia et al. 2006).

3.2.3 Response to Multiple Inducers

The interconnection of transduction pathways leads to the prediction that under multiple stresses, plant responses to a particular stress will be altered. These responses will be shaped by the interaction of the effects on gene regulation caused by individual stressors. Under the premise that crosstalk affects the expression of defence-related genes, the induction of VOCs by a particular individual stress should be affected when that stress occurs in the context of another stressor. Several studies have explored whether multiple stresses affect the VOC fingerprint of individual stressors (Table 3.1). There is evidence showing that the emission of VOCs under dual infestation is not merely a combination of those VOCs induced by individual stressors (Pierre et al. 2011b) and that the magnitude and direction of these changes depends on the plant species as well as the combination, incidence and timing of the two stresses (de Boer et al. 2008; Zhang et al. 2009). In addition to possible effects of crosstalk between signalling pathways on VOC biosynthesis and emission, the presence of a second stressor can affect the chemistry of plant tissues (Cardoza et al. 2003a; Kopper and Lindroth 2003; Rodriguez-Saona et al. 2010), altering herbivore feeding behaviour. This altered behaviour may have knock-on effects on VOC emissions (Zhang et al. 2009). Increased emissions of individual compounds have been reported for a number of combinations of piercing sucking and chewing herbivores (Delphia et al. 2007; de Boer et al. 2008) (Table 3.1). In the presence of phloem feeders, on the other hand, the emission of individual volatiles decreased (Rodríguez-Saona et al. 2003; Zhang et al. 2009; Schwartzberg et al. 2011), which fits with the fact that phloem feeders manipulate JA-dependent plant defences, as discussed above.

In the case of multiple stresses, temporal dynamics of plant responses to individual stresses are important in order to understand changes in VOC profiles due to crosstalk between signalling pathways. In the presence of phloem feeders, plant responses can take several days to develop (Zhang et al. 2009; Soler et al. 2012). For example, in lima bean (*Phaseolus lunatus*) an SA burst is observed only 7 days after infestation by *Bemisia tabaci* (Zhang et al. 2009). *B. tabaci* and *B. argentifolii* decrease *Tetranychus urticae*-induced emissions of (*E*)-β-ocimene in lima beans and *Spodoptera exigua*-induced emissions of myrcene, DMNT and TMTT in cotton plants when they start feeding on the plant prior to the other herbivore (Rodríguez-Saona et al. 2003; Zhang et al. 2009). In contrast, Moayeri et al. (2007) reported larger amounts of volatiles produced under 72 h of simultaneous attack by the aphid *Myzus persicae* and the spider mite *Tetranychus urticae* compared with single infestations (Table 3.1). Thus, variation in plant responses in the presence of multiple stresses may be shaped by the sequence of infestation and particularly the time elapsed between the stresses. The intensity of attack or stress can also affect the response of the plant and, in turn, the ecological roles of induced compounds (Zhang et al. 2009). In addition to herbivore feeding, oviposition can interfere with induced responses to herbivory. Oviposition by *Spodoptera frugiperda* can suppress herbivore-induced volatiles by conspecific larvae (Peñaflor et al. 2011), probably due to SA-mediated downregulation of JA-dependent genes (Bruessow et al. 2010). In systems involving stink bugs, oviposition and feeding damage sometimes do not interfere, and VOC blends are similar to those emitted after feeding alone (Colazza et al. 2004a; Michereff et al. 2011), though in some soybean cultivars oviposition does change herbivore-induced blends substantially (Moraes et al. 2008).

Compared to the growing interest in attack by multiple aboveground herbivores, little is known about how pathogens, belowground organisms and abiotic stresses can influence the emission of VOCs involved in multitrophic interactions. There is,

Table 3.1 Main findings of studies that have addressed the effect of multiple stresses on the emission of volatile organic compounds (VOCs)

Type of multiple stress	Plant	Stress agents[a]	Main findings[b]	Reference
Herbivores and abiotic stress	*Phaseolus lunatus* (Fabaceae)—lima bean	Ozone (O_3) *Tetranychus urticae*	Both O_3 and *T. urticae* induced GLVs and terpenoids Dual stress induced a similar blend as *T. urticae* alone	Vuorinen et al. (2004)
	Populus tremula × *Populus tremuloides* (Salicaceae)—hybrid aspen	O_3 *Epirrita autumnata*	*E. autumnata* alone induced several terpenoids Dual stress enhanced the emission of several herbivore-induced compounds (clone 110[c])	Blande et al. (2007)
	Populus tremula × *Populus tremuloides*—(Salicaceae)—hybrid aspen	O_3 *Phyllobius piri*	*P. piri* alone induced several terpenoids Dual stress enhanced the emission of several herbivore-induced compounds (clone 55[c])	Blande et al. (2007)
	Picea abies (Pinaceae)—Norway spruce	UV-B light *Hylobius abietis*	*H. abietis* alone induced several terpenoids Dual stress enhanced the emission of herbivore-induced MeSA	Blande et al. (2009)
	Solanum lycopersicum (Solanaceae)—tomato	O_3 *Bemisia tabaci*	Both single stress induced increased VOC emissions Dual stress caused a greater increase than the individual stresses	Cui et al. (2014)
Herbivores and pathogens	*Arachis hypogaea* (Fabaceae)—peanut	*Sclerotium rolfsii* *Spodoptera exigua*	Both *S. rolsfii* and *S. exigua* induced the emission of GLVs and terpenoids Dual stress enhanced the emission of *S. exigua*-induced compounds	Cardoza et al. (2002)
	Zea mays (Poaceae)—maize	*Setosphaeria turcica* *Spodoptera littoralis*	*S. turcica* induced greater emission of one terpenoid, while *S. littoralis* induced several GLVs and terpenoids Dual stress caused lowered emission of *S. littoralis*-induced compounds	Rostás et al. (2006)
	Brassica nigra (Brassicaceae)—black mustard	*Xanthomonas campestris pv. campestris* *Pieris brassicae*	Single damage by both *X. campestris* and *P. brassicae* induced distinct VOC blends compared to undamaged plants Dual stress caused a distinct VOC blend to be emitted, different from the individual damage	Ponzio et al. (2014)

(continued)

Table 3.1 (continued)

Type of multiple stress	Plant	Stress agents[a]	Main findings[b]	Reference
Aboveground herbivores—same feeding mode	*Brassica oleracea* (Brassicaceae)—cabbage	*Plutella xylostella*/ *Pieris rapae*	Each herbivore induced a distinct blend of VOCs, both dominated by terpenoids, such as DMNT Dual damage caused a larger emission of GLVs, dimethyl sulphide and a reduced amount of DMNT compared to single damage	Shiojiri et al. (2001)
Aboveground herbivores—different feeding mode	*Gossypium hirsutum* (Malvaceae)—cotton	*Bemisia argentifolii* *Spodoptera exigua*	During dual damage, whitefly nymphs decreased the amounts released after *S. exigua* damage, particularly of myrcene, DMNT and TMTT	Rodríguez-Saona et al. (2003)
	Nicotiana tabacum (Solanaceae)—tobacco	*Frankliniella occidentalis*/ *Heliothis virescens*	Single damage by *F. occidentalis* increased emission of (*E*)-β-ocimene and β-caryophyllene, while *H. virescens* damage increased 11 compounds Dual damage caused the induction of the 11 compounds induced by *H. virescens* and, additionally, α-humulene and caryophyllene oxide	Delphia et al. (2007)
	Capsicum annuum (Solanaceae)—sweet pepper	*Myzus persicae*/ *Tetranychus urticae*	*M. persicae* and *T. urticae* induced VOC blends with a few qualitative and quantitative differences Dual damage induced a similar blend, with the addition of two compounds: α-zingiberene and dodecyl acetate	Moayeri et al. (2007)
	Phaseolus lunatus (Fabaceae)—lima bean	*Spodoptera exigua*/ *Tetranychus urticae*	*T. urticae* and *S. exigua* induced distinct compounds Dual damage had a synergistic effect on the emission of individual compounds	de Boer et al. (2008)
	Cucumis sativus (Cucurbitaceae)—cucumber	*Spodoptera exigua*/ *Tetranychus urticae*	*T. urticae* and *S. exigua* induced the same compounds, but *S. exigua* caused emission in larger amounts Dual damage caused an additive effect on the emission of individual VOCs	de Boer et al. (2008)

	Phaseolus lunatus (Fabaceae)—lima bean	*Bemisia tabaci* *Tetranychus urticae*	Dual damage caused reduced emissions of (*E*)-β-ocimene compared to the emissions induced by *T. urticae*	Zhang et al. (2009)
	Zea mays (Poaceae)—maize	*Euscelidius variegatus* *Spodoptera littoralis*	Damage by *S. littoralis* and *E. variegatus* caused induction of different GLVs Dual damage caused the induction of a similar blend as *S. littoralis* alone	Erb et al. (2010)
	Vicia faba (Fabaceae)—broad bean	*Acyrthosiphon pisum* *Spodoptera exigua*	*A. pisum* had minor effects on VOC emission, while *S. exigua* induced several VOCs Upon dual damage, aphids suppressed the emission of *S. exigua*-induced VOCs	Schwartzberg et al. (2011)
	Brassica nigra (Brassicaceae)—black mustard	*Brevicoryne brassicae* *Pieris brassicae*	Dual attack did not cause a different VOC blend to be induced, despite minor quantitative differences in individual VOC amounts	Ponzio et al. (2014)
	Phaseolus lunatus (Fabaceae)—lima bean	*Mamestra brassicae* *Tetranychus urticae*	Dual attack caused plants to emit a very similar blend of VOCs as plants damaged by *T. urticae* alone	Menzel et al. (2014)
Above- and below-ground herbivores	*Zea mays* (Poaceae)—maize	*Diabrotica virgifera virgifera* (below-ground) *Spodoptera littoralis* (aboveground)	Aboveground, dual attack caused plants to emit a very similar blend of VOCs as plants damaged by *S. littoralis* Belowground, *S. littoralis* caused a reduction in the emission of *D. virgifera virgifera*-induced (*E*)-β-caryophyllene	Rasmann and Turlings (2007)
	Brassica nigra (Brassicaceae), black mustard.	*Delia radicum* (belowground) *Pieris brassicae* (aboveground)	Blends emitted after damage by *D. radicum* differed from blends emitted by plants damaged by either *P. brassicae* or dual damage. Blends emitted by plants damaged by *P. brassicae* and dual damage were correlated with higher levels of β-farnesene and dimehylnonatriene	Soler et al. (2007)

(continued)

Table 3.1 (continued)

Type of multiple stress	Plant	Stress agents[a]	Main findings[b]	Reference
	Brassica rapa subsp. *rapa* (Brassicaceae)—turnip	*Delia radicum* (belowground) *Pieris brassicae* (aboveground)	Dual damage caused a suppression of *D. radicum*-induced 4-mthylridecane, but an increase in 2-octen-1-ol, compared to single damage	Pierre et al. (2011b)
	Brassica rapa (Brassicaceae)	*Delia radicum* (belowground)/ *Pieris brassicae* (aboveground)	Aboveground, an increase was observed in methanethiol and methanol emission upon dual damage compared to single damage Belowground dual damage did not change patterns of emission of sulphur-based compounds compared to single damage by *D. radicum*	Danner et al. (2015)

Results are presented in chronological order. The list does not include the effects of damage by different stages of the same herbivore species, since this has been reviewed recently (Hilker and Fatouros 2015)

[a] The order of the stress agents in the list denotes also the sequence (different days) of the beginning of induction in multiple stress events. In the case of simultaneous induction (same day), stress agents are separated by a slash (/)

[b] The induced emissions from single factors refer to significant increases compared to control plants

[c] In this study, the authors quantified VOC emissions from two different clones grown in a FACE (free-air ozone concentration enrichment) facility in two summer seasons. Results presented in the table correspond to one clone over 1 year. Otherwise, there was no effect of ozone on herbivore-induced VOCs

however, evidence that simultaneous attack by a belowground herbivore and a shoot feeder can suppress important signalling compounds induced by aboveground herbivores in single infestations and cause emission of compounds not induced by any of the herbivores alone (Pierre et al. 2011a, b). Other studies have shown an increase in the amounts of certain compounds emitted aboveground (Danner et al. 2015) or no interference with the induction of VOCs by aboveground herbivores (Rasmann and Turlings 2007). On the other hand, aboveground herbivores can also interfere with VOCs induced by a herbivore feeding belowground (Rasmann et al. 2005; Rasmann and Turlings 2007) (Table 3.1). These results emphasise the importance of assessing dual infestation on spatially separated ecological interactions as one plant species under dual attack links many plant–herbivore interactions. Pathogens have been found to increase, decrease or not alter herbivore-induced VOCs (Cardoza et al. 2002; Rostás et al. 2006; Ponzio et al. 2014) (Table 3.1), whereas mycorrhizal fungi and endophytes can modify the release of herbivore-induced VOCs by herbivorous insects, either increasing or decreasing emissions (Fontana et al. 2009; Li et al. 2014). Decreased emissions in dual attack are expected to occur in the presence of a chewing herbivore and biotrophic pathogens. The latter can interfere with JA-dependent plant responses via activation of the SA pathway (Koornneef and Pieterse 2008).

Much less is known about how abiotic stresses affect herbivore-induced VOCs. This is surprising considering the effect that abiotic factors are known to have on VOC emissions (Gouinguené and Turlings 2002). A few studies have focused on simultaneous herbivory and ozone exposure, but the evidence is too scarce to generalise. Acute ozone exposure can result in an additive (Vuorinen et al. 2004) or synergistic (Cui et al. 2014) increase in VOC emission, but can also reduce (Himanen et al. 2009) or have no effect (Blande et al. 2007) on herbivore-induced VOCs (Table 3.1). This suggests that plant responses in the presence of ozone depend on dosage, exposure or plant–herbivore combination. Only a couple of studies have assessed the effect of UV-light on induced emissions, but virtually no effect of these light wavelengths on herbivore-induced VOCs was detected in field conditions (Winter and Rostás 2008; Blande et al. 2009).

3.3 Multiple Stresses and Plant-Mediated Interactions

VOCs emitted by plants are an important part of a plant's defensive phenotype, as discussed above. Changes in this defensive trait that impact other ecological interactions constitute trait-mediated indirect effects (Werner and Peacor 2003) between the inducing herbivore and the other species that interact with the plant and are now being considered in the context of trait-mediated indirect effects (Stam et al. 2014), but it is surprising that these interactions were not included sooner within this theoretical framework. Here we describe how VOCs induced by multiple stresses can mediate interactions between herbivores and the other organisms that interact with the plant.

3.3.1 Multiple Stresses, Competition and Facilitation

When feeding on a plant, a herbivore must confront not only the defences it induces, but also induced responses to other abiotic or biotic stresses, including other herbivores, which feed simultaneously or fed previously (Stam et al. 2014). Different stresses can occur simultaneously or sequentially, and this temporal component can lead to important ecological effects, since induction in response to one stress could potentially affect future attack by a series of herbivores. The timing of induced responses is extremely variable, but can start in a few hours and relax after a period of time, from a few days to several years (Karban and Baldwin 1997; Huntzinger et al. 2004; Karban 2011). Over the past 20 years, our understanding of the mechanisms underlying competitive interactions between herbivores has changed substantially. It is now clear that changes in plant quality can be as important as changes in plant abundance in mediating competition (Denno et al. 1995; Denno and Kaplan 2007). Furthermore, in some cases these induced changes can make the plant more susceptible to other herbivores, causing facilitation between herbivores using the same plant (Martinsen et al. 1998; Inbar and Gerling 2008; Soler et al. 2012; Ali et al. 2014). Plant-mediated interactions between herbivores should therefore be framed in a broad ecological context, avoiding the tempting simplification that all these interactions will be negative simply because the species are using a shared resource (Denno et al. 1995).

Before contact with the plant, perception of VOCs induced by a previous stress can cause reduced attraction of other phytophagous arthropods (Pallini et al. 1997; De Moraes et al. 2001; Delphia et al. 2007; Bleeker et al. 2009), possibly as a mechanism for avoiding competition or enemy-dense space. However, the plant can also become more apparent and attractive to other individuals (Landolt et al. 1999; Sarmento et al. 2011) acting as reliable cues of the presence of mating partners, better food quality (Cardoza et al. 2003b; Rodriguez-Saona et al. 2010) or plants with defences that have been overcome (Sarmento et al. 2011). In the case of VOCs induced by insect-vectored pathogens, this attraction may play an important role in pathogen dissemination (Ingwell et al. 2012; Mann et al. 2012).

Induction of different signalling pathways can modify plant traits such as leaf toughness or concentrations of secondary metabolites, which can affect host plant selection and performance of herbivores that subsequently colonise the plant. Females should oviposit on plants that optimise the fitness of their offspring, and reduced oviposition can be associated with lower performance on previously damaged plants (Inbar et al. 1999). However, increases in oviposition on previously stressed plants are also correlated with improved offspring development, a result of changes in primary or secondary plant chemistry (Cardoza et al. 2003a). Previous abiotic or biotic stress can increase, decrease or have no effect on herbivore oviposition preference [for reviews, see Rostás et al. 2003 (pathogen damage), Huberty and Denno 2004 (water stress) and Valkama et al. 2007 (ozone)]. However, the role of plant VOCs in host selection has not been addressed. Performance of *Pieris rapae*, *Plutella xylostella* and *Mamestra brassicae* was reduced on previously damaged plants, but *P. xylostella* laid more eggs on damaged plants (Poelman et al. 2008). Since oviposition selection is a balance between food quality and predation risk (Shiojiri et al. 2002), we need a better understanding of how multitrophic context affects oviposition choices. For example, *T. urticae* prefers to oviposit and settle on whitefly-infested plants over undamaged lima bean plants (Zhang et al. 2009). The role of VOCs is likely to be crucial, as infestation by whiteflies can interfere with prey location by predatory mites (Zhang et al. 2009); thus, plant selection could allow *T. urticae* to escape from natural enemies.

Induced plant defence can persist, affecting the herbivore community on the plant, often asymmetrically. Milkweed (*Asclepias syriaca*) is attacked by several different herbivores during the growing season, and the sequence of damage can alter the susceptibility to each herbivore, creating highly asymmetric effects. Damage by the weevil *Rhyssomatus lineaticollis* makes milkweed less susceptible to the monarch butterfly, *Danaus plexippus*, later in the season, but plants damaged by the monarch are more susceptible to damage by the weevil, as well as other herbivores (Van Zandt and Agrawal 2004a, b). On the other hand, damage by the aphid *Aphis nerii* makes the plant more susceptible to the monarch, while damage by the monarch makes the plant more resistant against the aphid (Ali et al. 2014). *Solanum dulcamara* attacked by three species of chrysomelid beetles (two flea beetles and a tortoise beetle) deploys different defensive responses against each herbivore, and previous damage by flea beetles decreases tortoise beetle occurrence, driven primarily by reduced oviposition preference. Damage by tortoise beetles, however, increases flea beetle occurrence on damaged plants relative to controls (Viswanathan et al. 2005, 2008). Interestingly, the season-long effect of induction on these herbivores is strongly determined by the first herbivore to attack the plant and is not altered by damage by subsequent herbivores (Viswanathan et al. 2007).

These asymmetric effects occur between arthropod herbivores of similar sizes. Asymmetries are expected to become more severe with greater differences between attackers in size and feeding mode. Pathogens can affect herbivore performance through activation of both JA- and SA-dependent responses, though different pathogens can affect herbivores in distinct ways, and effects can be reciprocal (Thaler et al. 2010). Severe asymmetries, both positive and negative, are expected between mammalian and insect herbivores (Martinsen et al. 1998; Gomez and Gonzalez-Megias 2002, 2007). Induced VOCs could play important roles in these plant-mediated interactions. Rostás et al. (2013) demonstrated that gall-induced VOCs are avoided by goats, thus protecting the developing larvae. We expect that mammal-induced VOCs have important effects on insect herbivores, potentially through the emission of large amounts of GLVs, and mammal saliva should also interact with plant defence responses, though, to our knowledge, no study has been carried out with mammal-induced plant defences.

3.3.2 Multiple Stresses and Tritrophic Interactions

Plant odour plumes are extremely variable (Beyaert and Hilker 2014), and a challenge for foraging insects is to separate the signal from the noise in these highly variable chemical mixtures. It appears that insects and mites discard most of the variation as background noise, while reliable changes in VOC blends indicate the presence of a given herbivore species on the plant (Pareja et al. 2009; Bruce and Pickett 2011; McCormick et al. 2012). Damage to the plant by multiple attackers could, in theory, cause substantial noise that herbivore natural enemies need to process. However, we are discovering that, even with multiple damage events, these arthropods are capable of deciphering the signal indicating host or prey presence on a particular plant (Table 3.2).

The simplest form of multiple damage events occurs when the same herbivore induces the plant in different ways. This is particularly relevant in the case of oviposition-induced and herbivory-induced cues. Egg parasitoids need to find an ephemeral and inconspicuous host and are known to use induced plant VOCs (Fatouros et al. 2008a; Hilker and Fatouros 2015). Some species of egg parasitoid respond to oviposition-induced VOCs resulting from a plant response triggered by cues in the oviposition secretion of the female laying the eggs (Hilker and Meiners 2010; Hilker and Fatouros 2015) or even by male-derived pheromones passed on to the female during copulation (Fatouros et al. 2008b). In these cases, induction by oviposition alone is sufficient to attract the parasitoid to the plant. However, in several systems the picture is more complicated. The parasitoid of pentatomid eggs, *Trissolcus basalis,* is only attracted to plants induced simultaneously by both adult feeding and oviposition (Colazza et al. 2004a, b). In a similar system, *Telenomus podisi*, which also parasitizes stink bug eggs, is attracted to soybean plants that suffered feeding damage by adults and also to plants damaged by adults and subjected to oviposition (Michereff et al. 2011). However, in some soybean cultivars attraction to herbivore-damaged plants is switched off if plants are also subjected to oviposition (Moraes et al. 2008).

When we consider damage by different herbivore species, a greater variety of plant-mediated effects occur since there is a greater number of possible combinations of induction. The plant recognises each stressor differently due to saliva and feeding mode. Further, natural enemies differ in host or prey range, and this affects their responses to induced VOCs (Vet and Dicke 1992; Steidle and van Loon 2003). Parasitoids have a narrower prey range than predators, and multiple damage events are expected to interfere more with their olfactory responses (de Rijk et al. 2013). A few studies have confirmed this expectation, showing reduced attraction of parasitoids to multiply damaged plants (Shiojiri et al. 2000; Rasmann and Turlings 2007; Soler et al. 2007; Yamamoto et al. 2011) (Table 3.2). However, some studies have shown an increased response by parasitoids to plants damaged by both host and non-host herbivores (Shiojiri et al. 2000, 2001; Rodríguez-Saona et al. 2005; Bukovinszky et al. 2012), pathogens (Cardoza et al. 2003a) and ozone stress (Cui et al. 2014). Finally, several studies have demonstrated that multiple stresses have

Table 3.2 Main findings of studies that have addressed the effect of multiple stress on the attraction of herbivore natural enemies

Type of multiple stress	Plant	Stress agents[a]	Natural enemy[b]	Main findings	Reference
Herbivores and abiotic stress	*Phaseolus lunatus* (Fabaceae)—lima bean	O_3 *Tetranychus urticae*	*Phytoseiulus persimilis* (Pr)	The predator preferred *T. urticae*-damaged plants regardless of whether plants were grown in ozone or not. The odours induced by ozone are not attractive to the predator	Vuorinen et al. (2004)
	Solanum lycopersicum (Solanaceae)—tomato	O_3 *Bemisia tabaci*	*Encarsia formosa* (Pa)	The parasitoid preferred plants under either stress over undamaged plants, but had an enhanced response to dual stress over both types of single stress	Cui et al. (2014)
Herbivores and pathogens	*Arachis hypogaea* (Fabaceae)—peanut	*Sclerotium rolfsii* *Spodoptera exigua*	*Cotesia marginiventris* (Pa)	Wasps orientated to, and landed more on, previously herbivore-damaged plants that had previous infection with *S. rolfsii* compared to herbivore-damaged plants	Cardoza et al. (2003a)
	Zea mays (Poaceae)—maize	*Setosphaeria turcica* *Spodoptera littoralis*	*Microplitis rufiventris* (Pa)	Wasps did not discriminate between the odours induced by the host alone and by the host and fungus together. The odours induced by the fungus were not attractive to the wasps	Rostás et al. (2006)
	Zea mays (Poaceae)—maize	*Setosphaeria turcica* *Spodoptera littoralis*	*Cotesia marginiventris* (Pa)	Wasps did not discriminate between the odours induced by the host alone and by the host and fungus together. The odours induced by the fungus were not attractive to the wasps	Rostás et al. (2006)
	Brassica nigra (Brassicaceae)—black mustard	*Xanthomonas campestris pv. campestris* *Pieris brassicae*	*Cotesia glomerata* (Pa)	Wasps preferred the odours of host-infested plants in the presence of the bacterium over those from control plants. The wasps also preferred the odours induced by dually infested plants over those by host alone	Ponzio et al. (2014)

(continued)

Table 3.2 (continued)

Type of multiple stress	Plant	Stress agents[a]	Natural enemy[b]	Main findings	Reference
Aboveground herbivores—same feeding mode	*Brassica oleracea* (Brassicaceae)—cabbage	*Plutella xylostella* (host) *Pieris rapae*	*Cotesia plutellae* (Pa)	Wasps discriminated between odours induced by host and non-host, and they preferred plants damaged by the host over dual-infested plants	Shiojiri et al. (2001)
	Brassica oleracea (Brassicaceae)—cabbage	*Plutella xylostella* *Pieris rapae* (host)	*Cotesia glomerata* (Pa)	Wasps did not discriminate between odours induced by host and non-host, and they preferred plants damaged by both a host and a non-host over plants damaged by the host alone	Shiojiri et al. (2001)
	Brassica oleracea (Brassicaceae)—cabbage	*Plutella xylostella* (host) *Pieris rapae*	*Cotesia vestalis* (=*Cotesia plutellae*) (Pa)	The wasp preferred plants damaged by the host over dual-damaged plants, but this preference depended on density and stage of the non-host	Yamamoto et al. (2011)
	Brassica oleracea (Brassicaceae)—cabbage	*Pieris rapae* (host) *Mamestra brassicae*	*Cotesia glomerata*	Plants suffering dual damage were more attractive than those under single damage by either species. Parasitoids had a reduced attack rate in mixed patches. Parasitism of *P*. *rapae* in the field was reduced on plants with dual damage	Bukovinszky et al. (2012)
	Capsicum annuum (Solanaceae)—sweet pepper	*Myzus persicae* *Aphis gossypii*	*Cycloneda sanguinea* (Pr)	The predator did not differentiate between odours of singly damaged plants and plants damaged by both aphid species when damage was simultaneous. A specific combination of sequential damage increased predator attraction	Oliveira and Pareja (2014)
Aboveground herbivores—different feeding mode	*Solanum lycopersicum* (Solanaceae)—tomato	*Macrosiphum euphorbiae* *Spodoptera exigua* (host)	*Cotesia marginiventris* (Pa)	Wasps responded more rapidly and landed more frequently on dual-infested plants	Rodríguez-Saona et al. (2005)

	Capsicum annuum (Solanaceae)—sweet pepper	*Myzus persicae* *Tetranychus urticae*	*Macrolophus caliginosus* (=*M. melanotoma*) (Pr)	The predatory bug preferred the odours of dual-infested plants over those induced by *T. urticae* or *M. persicae* alone	Moayeri et al. (2007)
	Brassica chinensis vr. *pekinensis* (Brassicaceae)—Chinese cabbage	*Myzus persicae* (host) *Plutella xylostella*	*Diaeretiella rapae* (Pa)	Wasps showed equal preference for aphid-damaged plants when compared to plants damaged by aphids and caterpillars	Agbogba and Powell (2007)
	Phaseolus lunatus (Fabaceae)—lima bean	*Spodoptera exigua* *Tetranychus urticae* (prey)	*Phytoseiulus persimilis* (Pr)	The predatory mite preferred the odours of dual-damaged plants over those induced by *T. urticae* alone	de Boer et al. (2008)
	Cucumis sativus—(Cucurbitaceae)—cucumber	*Spodoptera exigua* *Tetranychus urticae* (prey)	*Phytoseulus persimilis* (Pr)	The predatory mite preferred the odours of dual-damaged plants over those induced by *T. urticae* alone	de Boer et al. (2008)
	Phaseolus lunatus (Fabaceae)—lima bean	*Tetranychus urticae* (prey) *Bemisia tabaci*	*Phytoseiulus persimilis* (Pr)	The predatory mite preferred the odours induced by *T. urticae* alone over those induced by dual infestation. Reduction of attraction caused by *B. tabaci* is density dependent	Zhang et al. (2009)
	Zea mays (Poaceae)—maize	*Spodoptera littoralis* (host) *Euscelidius variegatus*	*Cotesia marginiventris* (Pa)	Wasps showed equal preference for *S. littoralis*-damaged plants when compared to plants damaged by both herbivores	Erb et al. (2010)
	Solanum lycopersicum (Solanaceae)—tomato	*Tuta absoluta* *Bemisia tabaci*	*Nesidiocoris tenuis* (Pr)	The predator did not prefer dual-infested plants over single-infested plants. This response did not change after experience with dually-infested plants	Lins et al. (2014)

(continued)

Table 3.2 (continued)

Type of multiple stress	Plant	Stress agents[a]	Natural enemy[b]	Main findings	Reference
	Solanum lycopersicum (Solanaceae)—tomato	*Tuta absoluta* *Bemisia tabaci*	*Macrolophus pygmaeus* (Pr)	The predator preferred dual-infested plants when naïve. However, after experience with dual-infested plants the predator lost this preference	Lins et al. (2014)
	Brassica nigra (Brassicaceae)—black mustard	*Brevicoryne brassicae* *Pieris brassicae* (host)	*Cotesia glomerata* (Pa)	The parasitoid was able to discriminate between uninfested and host-infested plants in the presence of aphids regardless of aphid density. The wasp was equally attracted to odours induced by the host alone and host in the presence of aphids	Ponzio et al. (2014)
	Phaseolus lunatus (Fabaceae)—lima bean	*Mamestra brassicae* *Tetranychus urticae* (prey)	*Phytoseiulus persimilis* (Pr)	The predatory mites were attracted to odours induced by spider mites alone or previously induced by caterpillar oral secretions	Menzel et al. (2014)
	Brassica juncea (Brassicaceae)	*Myzus persicae* (host) *Plutella xylostella*	*Aphidius colemani* (Pa)	Wasps did not distinguish between the odour from plants damaged by both herbivores and those from plants damaged by the host alone	Silva et al. (2016)
	Brassica juncea (Brassicaceae)	*Myzus persicae* (preferred prey) *Plutella xylostella*	*Chrysoperla externa* (Pr)	The predator preferred the odour from plants damaged by both herbivores over the odour from plants damaged by the aphid alone	Silva et al. (2016)
Above- and below-ground herbivores	*Zea mays* (Poaceae)—maize	*Diabrotica virgifera virgifera* *Spodoptera littoralis* (host)	*Cotesia marginiventris* (Pa)	Wasps were more attracted to the odours induced by *S. littoralis* alone over the odours induced by dual infestation. Experience in dual infestation shifted this preference	Rasmann and Turlings (2007)

	Zea mays (Poaceae)—maize	*Diabrotica virgifera virgifera* (host) *Spodoptera littoralis*	*Heterorhabditis megidis* (EP)	*H. megidis* preferred the odours of plants damaged by *D. virgifera virgifera* alone over the odours of plants in dual infestation	Rasmann and Turlings (2007)
	Brassica nigra (Brassicaceae)—black mustard	*Delia radicum* *Pieris brassicae* (host)	*Cotesia glomerata* (Pa)	Wasps did not discriminate between odours induced by the host alone or in the presence of the root feeder. They were repelled by the odours emitted by *D. radicum*-infested plants alone	Soler et al. (2007)
	Brassica rapa subsp. *rapa* (Brassicaceae)—turnip	*Delia radicum* (host) *Pieris brassicae*	*Trybliographa rapae* (Pa)	The preference of the wasps for *D. radicum*-infested plants is lost in the presence of the aboveground feeder	Pierre et al. (2011a)

Results are presented in chronological order. The list does not include the effects of damage by different stages of the same herbivore species, since this has been reviewed recently (Hilker and Fatouros 2015)

[a] For predators that do not prey on both herbivores, the prey is indicated. For parasitoids and entomopathogens, the host is indicated. If a prey is not indicated, both herbivores can serve as prey for that particular predator

[b] The abbreviation next to the natural enemy indicates whether it is a predator (Pr), a parasitoid (Pa) or an entomopathogen (EP)

no effect on parasitoid attraction to damaged plants (Rostás et al. 2006; Silva et al. 2016; Agbogba and Powell 2007; Himanen et al. 2009; Erb et al. 2010; Ponzio et al. 2014) (Table 3.2). Since herbivore performance can determine whether it is a good quality host for parasitoids (Van Emden and Kifle 2002), induced defences can alter parasitoid performance indirectly (Harvey et al. 2003). It will be interesting to investigate whether increased parasitoid responses to plants under multiple stress correspond to increases in the quality of hosts that feed on multiply damaged plants.

Most studies with predators of arthropod herbivores have shown increased attraction to plants suffering multiple stresses (Moayeri et al. 2007; Silva et al. 2016; Lins et al. 2014) though a few species are not affected (Lins et al. 2014; Oliveira and Pareja 2014). Oliveira and Pareja (2014) studied both simultaneous and sequential damage by two aphid species on pepper and demonstrated that, although simultaneous damage did not alter the attraction of the ladybird *Cycloneda sanguinea*, a specific combination of sequential damage resulted in increased attraction. The predatory mite *Phytoseiulus persimilis* has been the most intensively studied species and demonstrates the complexity of responses that a single species can show to different VOC contexts. *P. persimilis* showed enhanced responses to plants damaged by its prey *T. urticae* and by non-prey species *S. exigua* (de Boer et al. 2008). However, sequential induction in lima bean by *M. brassicae* oral secretion and *T. urticae* did not enhance *P. persimilis* attraction (Menzel et al. 2014). A study has also shown reduced attraction with increasing whitefly density on the plant (Zhang et al. 2009). Combined ozone stress and *T. urticae* damage did not disrupt *P. persimilis* attraction (Vuorinen et al. 2004) (Table 3.2). This context dependence of multiple stresses is likely to be common, and the challenge is to understand the common features of cases where indirect defence is enhanced, hindered or unaffected. It is not yet clear whether some of the contrasting results are due to differential prey suitability of the different herbivores. A complicating factor is that many predators rely on learning to find suitable prey. Therefore, responses by naïve individuals could be altered by experience in different VOC contexts and in response to prey suitability in different patches (Bukovinszky et al. 2012).

Despite the few studies testing the effects of multiple damage events, patterns do emerge that warrant further study. The clearest involve responses to plants damaged by two herbivores of different feeding guilds. Contrary to expectations, studies addressing this found no reduction in responses and revealed tritrophic signals that withstand multiple attackers (Agbogba and Powell 2007; Moayeri et al. 2007; Erb et al. 2010; Ponzio et al. 2014; Silva et al. 2016) and can even be enhanced (Rodríguez-Saona et al. 2005; de Boer et al. 2008; Lins et al. 2014). Few studies have shown reduced attraction (Zhang et al. 2009). Damage by herbivores of similar feeding guilds gives clearer results, either enhancing or reducing attraction (Shiojiri et al. 2000, 2001; Yamamoto et al. 2011; Bukovinszky et al. 2012; Oliveira and Pareja 2014). The same applies to plants damaged by foliar and root herbivores (Rasmann and Turlings 2007; Soler et al. 2007). Though these patterns could provide a framework for future work, they must be cautiously interpreted for several important reasons. First, the number of studies is still extremely small, and future work could change the observed patterns. Second, the systems studied are very narrow; Brassicaceae and *Cotesia* parasitoids dominate. Finally, as pointed out by Ali and Agrawal (2012), phylogenetically controlled comparisons need to be incorporated in order to determine whether patterns reflect ecologically mediated selection pressures or phylogenetic constraints.

3.3.3 Induced Defences and Pollinators

Plants depend on effective communication with their animal pollinators to maintain pollen flow between plant individuals. This communication is mediated by floral display, and herbivory to vegetative parts can interfere with this display and with pollinator visitation (Lehtilä and Strauss 1997; Mothershead and Marquis 2000; Poveda et al. 2003, 2005). Chemical signals, in particular floral VOCs, are recognised as key players in this interaction (Raguso 2008, 2009; Lucas-Barbosa et al. 2011). Since floral VOCs are synthesised through biochemical pathways that are also involved in plant defence (Dudareva et al. 2013), it is reasonable to expect that induced defences against attackers affect floral chemistry. Direct herbivory on flowers can alter floral scent (Zangerl and Berenbaum 2009), and, in recent years, the effect of leaf damage on floral VOCs has received increasing attention, revealing effects that appear to be herbivore dependent. Feeding by the *Brassica*-specialist aphid *Lipaphis erysimi* practically shuts down floral VOC emission in white mustard, but the extreme generalist *Myzus persicae* caused a less pronounced suppression (Pareja et al. 2012). Some of the first studies addressing caterpillar and mechanical (simulated) damage did not detect a reduction in floral VOC emission (Effmert et al. 2008; Theis et al. 2009; Pareja et al. 2012), and recently an increase in floral VOC emission was reported after caterpillar damage (Cozzolino et al. 2015). However, certain herbivores might not affect the quantity of VOCs emitted but rather change diel patterns of emission (Kessler et al. 2010) or blend composition (Bruinsma et al. 2014). As for other aspects of induced defence (Ali and Agrawal 2012), feeding mode could be fundamental in determining the effects of herbivory on floral VOCs.

Changes in floral VOCs after herbivory can reduce pollinator visits in the field and seed set by the plant (Kessler et al. 2011; Lucas-Barbosa et al. 2015). Some ecological effects of induction can be even more complex. In *Nicotiana attenuata,* caterpillar herbivory causes a complete change in flowering biology, with changes in VOC emission and flower opening times, which alters the relative importance of different pollinators (Kessler et al. 2010). In *Silene latifolia*, herbivore damage increases seed set, but only in plants exposed to night-active pollinators (Cozzolino et al. 2015). So far very few studies have addressed trait-mediated interactions between herbivores and pollinators, so there is much to be learned about the ecological effects of VOC changes. The physiological costs of herbivory on reproductive allocation could be compounded by ecological costs in reduction of pollinator visitation. On the other hand, reduced visitation time and induced toxins in the nectar could increase efficiency of pollen transfer (Lucas-Barbosa et al. 2011; Bruinsma et al. 2014). Future studies addressing effects on plant fitness should begin to tease apart physiological and ecological costs of herbivore-induced plant defences. Further, we need to incorporate the effects of multiple damage events on floral VOC production, since plants in nature need to flower and attract pollinators after a lifetime of coping with different stresses.

3.4 Conclusions and Future Directions in the Community Ecology of Plant Volatiles

We have discussed some of the behavioural and chemical aspects of induced plant defence against single and multiple stresses. With this knowledge, we are now in a position to begin to address the ecological roles induced plant VOCs play in more complex ecological settings. Here we highlight questions relating to multiple damage events that are of particular interest to us and that we believe are understudied. We focus on stress caused by herbivores because those are the systems we work with, and not because other types of stress are less important or interesting. We believe that most of the questions are easily transferable.

Induced plant responses to individual or multiple stresses can change plant phenotypes in ways that can have important community consequences. Thus, the induced changes in plants have the potential to mediate many indirect effects in ecosystems. Plants as mediators of indirect effects have received increased attention over the last decade (Ohgushi 2005, 2008; Ohgushi et al. 2007, 2012), and plant VOCs are beginning to be included within this framework (Poelman et al. 2008; Lucas-Barbosa et al. 2011; Dicke et al. 2012; Stam et al. 2014). An important avenue of research will be integrating the accumulated behavioural and chemical information with the conceptual framework of indirect effects in ecosystems (Wootton 1994; Abrams 1995; Werner and Peacor 2003; Schoener and Spiller 2012). Of particular interest are questions about how phenotypic plasticity in plant VOCs after single and multiple stresses affects other herbivores, mutualists, predators and parasitoids under field conditions, and how damage by two stressors percolates through webs with different topologies (Schoener and Spiller 2012). Increased predator or parasitoid attraction to plants that suffer multiple stresses could create enemy-dense space for a given herbivore (Biere et al. 2002). Reduced attraction could, on the other hand, create relative enemy-free space (Stam et al. 2014), and plant responses to multiple damage events could change relative patterns of predation and parasitism in a given community. Furthermore, spatial and temporal variation in natural enemy community composition could result in very different tritrophic effects of induced plant defence and variable selective pressures on these defences (Thompson 2005). A community perspective, incorporating multiple herbivores and multiple natural enemies in both laboratory and field studies, is beginning to address these questions (Poelman et al. 2008; Bukovinszky et al. 2012; Tack et al. 2013; Silva et al. 2016; Stam et al. 2014).

Induced responses to abiotic stress could also be extremely important in generating heterogeneity in plant–arthropod or plant–pathogen interactions over relatively small spatial and temporal scales. In addition to inducing VOCs, plants respond to abiotic stress with the accumulation of other secondary metabolites (for example flavonoids under UV-light exposure; Winkel-Shirley 2002) that have significant roles in plant–arthropod interactions, and effects that may percolate towards higher trophic levels. In nature, extreme abiotic conditions of light or drought are likely to occur several times during a plant's lifespan. The process of hardening (Bruce et al. 2007) may play an important role in subsequent responses to stress. To what extent the physiological and biochemical changes in the plant responsible for this hardening play a role in plant responses to further plant–arthropod or plant–pathogen interactions is unknown. The process of hardening and priming is normally dismissed in laboratory experiments, though priming of plants by herbivory is known to increase VOC emissions upon subsequent attack (Ton et al. 2006). Immunisation of plants to one herbivore upon mild damage by another species has also been reported (Kessler and Baldwin 2004). Future abiotic conditions are expected to become harsher for plants. Droughts, increased ozone concentrations and high UV-B radiation are likely to become more frequent in the future. The research field is now ready to study induced plant defence from a community perspective, including multiple herbivores, plant diversity, mutualists and variation in the abiotic environment (Wäschke et al. 2013; Pierik et al. 2014).

The responses of predators and parasitoids to plants under multiple stresses could be highly influenced by associative learning and other behavioural plasticity. There is evidence that the response of parasitic wasps to plant odours in the presence of another inducer can be modified through associative learning (Rasmann and Turlings 2007). Therefore, natural enemies might be able to overcome signal interference due to multiple stresses through learning. Further, if host or prey (herbivore) quality varies through changes in herbivore performance after multiple damage (see above), natural enemies could learn to associate high-quality hosts with distinct plant odours. Studies using naïve individuals do not consider these effects, and future work should begin to address whether this behavioural plasticity is used by natural enemies to increase foraging efficiency when plants are subjected to multiple stresses.

The majority of studies cited above come from temperate systems, in particular domesticated plants (Chen et al. 2015). The tropics are home to the majority of the Earth's biological diversity, yet we know very little about chemically mediated multitrophic interactions in agricultural, let alone in natural, tropical systems. These regions are at the forefront of global efforts to stem the loss of biodiversity and ecosystem services, so focus on these systems is long overdue. Tropical ecosystems can have an enormous diversity of plant–herbivore combinations in both space and time. Therefore, individual plants are likely to be damaged by more attackers than in temperate ecosystems, and the density of an individual plant–herbivore combination is likely to be much lower. Highly specialised natural enemies, for example most parasitoids, will face a more difficult task to locate a specific plant–herbivore combination, and plant defence and parasitoid responses might reflect this. Another important characteristic of many tropical systems is the high abundance of generalist predators such as wasps and, most notably, ants (Rico-Gray and Oliveira 2007). Plant signalling in these systems might have evolved with these extremely generalist predators as a major selective pressure, and induced plant responses could reflect this (Vet and Dicke 1992; Steidle and van Loon 2003). It will be interesting to begin to understand how VOC webs map onto these systems and to understand the evolution of indirect plant defence in these hyper-diverse systems. Understanding ecological interactions in tropical agricultural systems is also increasingly important for obtaining food security and enhancing conservation efforts. Most research in chemical ecology in South America, for example, has focused on crops important for large-scale industrial farming, such as soybean, maize and cotton, where sustainable, knowledge-intensive (as opposed to input-intensive) methods are unlikely to be implemented. In small-scale, diversified agroecosystems, ecological interactions are much more important for pest control (Perfecto and Vandermeer 2010, 2015). Chemical ecologists in the tropics should begin to shift their focus from industrial crops to traditional crops, where ecological complexity will have an important role in sustainable food production (Cook et al. 2007; Khan et al. 2014).

Induced plant VOCs have taken centre stage in chemical ecology and are now being integrated into general ecology theories of community organisation and co-evolutionary dynamics (Dicke 2009; Hare 2011; Stam et al. 2014). We have made significant inroads in our understanding of the behavioural and chemical processes that involve plant VOCs, but we are only beginning to scratch the surface of the ecological consequences of induced VOCs, and how they might modulate community structure. The ecological effects of multiple stresses involve many different players, in many different combinations. We now need to embrace the complexity of chemically mediated ecological interactions in order to understand the role induced VOCs play in both natural and agricultural ecosystems.

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Chapter 4
Measuring Rapid Changes in Plant Volatiles at Different Spatial Levels

Pawel K. Misztal

Abstract The majority of volatile chemical measurements related to plant communication processes have been conducted at relatively small spatial scales. Relatively little is known about how volatile-mediated signalling functions at larger scales, such as large plant, ecosystem or region. To understand these issues, real-time measurement of volatile organic compounds (VOC), which has been successfully used in the atmospheric science community for almost two decades, is required. When VOCs and vertical wind speed are measured at sufficiently high temporal resolution, eddy correlation techniques can be used to provide direct information about the ecosystem biosphere–atmosphere exchange. These very fast measurements can reflect the true dynamics of the concentrations of key semiochemicals, which could otherwise be averaged out over longer time periods. Furthermore, they allow for direct measurement of their ecosystem net flux from a well-defined area, which enables a holistic understanding of a habitat's chemistry and physics. This chapter is intended to inspire chemical ecologists to view the bigger picture in chemical communication by applying real-time measurement approaches at larger scales. This chapter presents the principles of real-time measurements of semiochemicals by PTR-MS and the eddy covariance technique along with examples of their current and potential applications in field measurements.

Glossary of Technical Terms and Acronyms

GC-MS	Gas chromatography mass spectrometry
PBL	Planetary boundary layer
PTR-MS	Proton transfer reaction mass spectrometry

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QMS	Quadrupole mass spectrometer (a detector used in a classic PTR-MS resolving ions at a unit mass resolution with 1 Da, e.g. 153 for protonated methyl salicylate).
SPME	Solid-phase micro-extraction fibres.
SQT	Sesquiterpenes
TOF	Time-of-Flight detector (a detector used in a novel type of PTR-MS resolving an exact mass within ~1 mDa, e.g. 153.055 for protonated methyl salicylate)
VOC	Volatile organic compounds
BVOC	Biogenic VOC

4.1 Introduction

Chemical interactions across and within different trophic levels are highly complex and reliant upon volatile cues (Dudareva et al. 2004). Some of these cues exist as characteristic scents which can be a single chemical as is the case with many insect alarm pheromones (or allomones) (e.g. Gibson and Pickett 1983), but other scents comprise complex blends of chemicals at very specific ratios, as is the case with many insect sex pheromones (McFrederick et al. 2009). Thousands of compounds have been identified from floral scents (Knudsen et al. 2006). Understanding chemical communication across all trophic levels within an ecosystem is challenging due to the spatial and temporal patterns of chemical cues, the presence of interfering or overlapping compounds and/or a highly oxidative/nitrosative environment [e.g. O_3, NO_x ($NO_x \equiv NO + NO_2$) from atmospheric pollution]. A highly sensitive semiochemical flux measurement system at a larger scale and with high temporal resolution would be desired for tracking these ensemble interactions (Fig. 4.1).

Different parts of a plant, including flowers, leaves, stem and bark can emit the same or different volatile chemicals, which can differ in their roles as signals depending on the receiving organism. Estragole, for example, is emitted from oil palm flowers, which attracts the oil palm's pollinating weevil (Misztal et al. 2010), but is also emitted from pine needles and bark resins, which mostly deters pine bark beetles (Bouvier-Brown et al. 2009). The chemical make-up of plants characterised by different phenylpropanoid contents in leaves varies according to species, and some of these compounds (e.g. eugenol and methyl eugenol) can serve as attractants for certain insects, including pollinators (often dependent on the dose), while acting as potent deterrents for some herbivores (Tan and Nishida 2012). While interactions mediated by benzenoid semiochemicals are well defined for specific biological systems, the functions of those biogenic benzenoids observed at the ecosystem scale are less clear; nevertheless, they could provide important signals and hence their measurement could be valuable from a chemical ecology perspective (Misztal et al. 2015). While it would be challenging to understand ecosystem functioning by looking at individual leaves or even plants, ecosystem-scale measurements can be

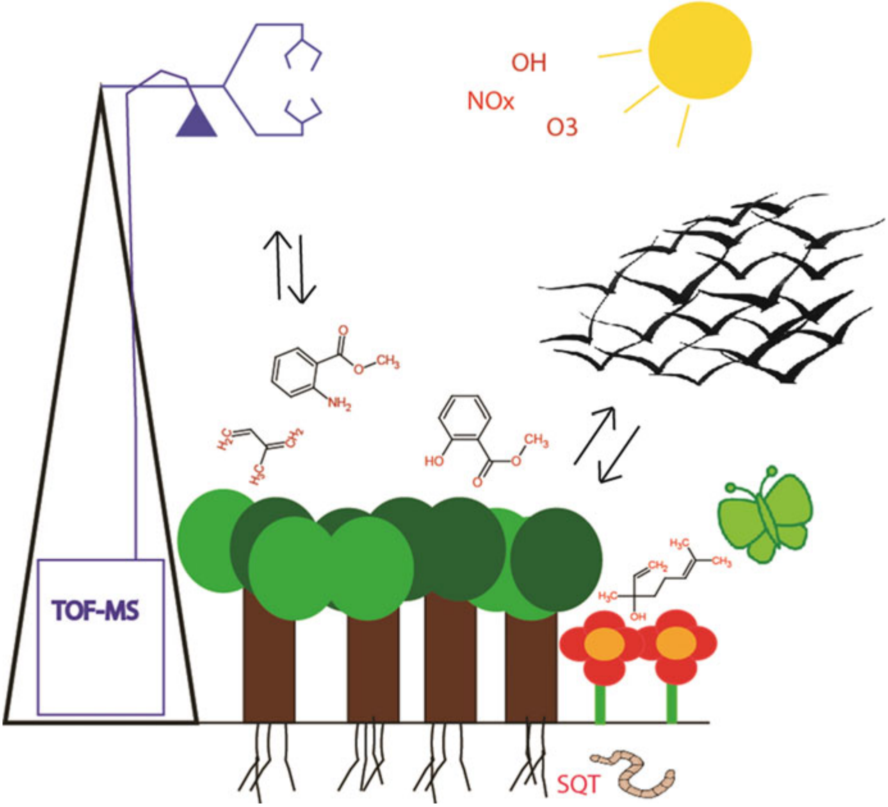

Fig. 4.1 Conceptual schematic of multitrophic volatile-mediated interactions sensed at an ecosystem scale. The eddy covariance system coupled with a mass spectrometer (e.g. based on a Time-of-Flight detector) measures an ensemble of ecosystem originated volatiles that are involved in various multitrophic interactions

more representative of stress or defence signal release on a larger scale and can be useful for modelling and simulation of the complexity of biogenic VOC (BVOC) emitted by diverse vegetation (Guenther 2013). Globally, isoprene is the single most abundant BVOC (Guenther et al. 1995); it is a powerful antioxidant, but its role in chemical communication is not well understood (Laothawornkitkul et al. 2008). The mix of observed atmospheric chemical constituents is extremely complex (Goldstein and Galbally 2007), so deciphering and making biological sense of this complexity is a fascinating challenge for chemical ecology and related sciences.

In the field of chemical ecology, a huge amount of information has been obtained in terms of the emissions that specific plant parts and small enclosable whole plants emit under certain stresses. Plants are, however, exposed to numerous biotic and abiotic stressors, which have often been explored in isolation (although recent attempts have been made to start addressing that shortcoming, see Chap. 3).

Moreover, big challenges remain in extrapolating lab results to field conditions, where the environment is vastly more complex. In line with earlier limitations for the field, a number of contemporary burning questions arise, including:

1. What is the variability of stress-related or defence semiochemicals (e.g. methyl salicylate) at the ecosystem scale?
2. What are the diurnal and seasonal trends of common pollinator attractants above crops and forests?
3. How does pollution affect the lifetimes and transport of communication signals?
4. How do ecosystems adapt to an anthropogenically influenced environment?

The methods described in the rest of this chapter may well be used to address these questions and open up possibilities for research that crosses the disciplines of environmental science and chemical ecology.

4.2 Tools Commonly Used for Measuring Volatiles

The common methods related to GC measurements of plant volatiles have been described in detail elsewhere (e.g. Tholl et al. 2006), but real-time approaches combining the recently developed PTR-TOF-MS instrument in combination with micrometeorological approaches have not yet been widely advocated for tracking chemical communication at ecosystem scales. In the past, measurements of VOCs in ambient air usually reported dozens of compounds, but PTR-TOF-MS enables a comprehensive look at how hundreds of ions vary in real time (Park et al. 2013a).

A common tool for measurement of plant volatiles is to collect the VOCs in the headspace of a plant enclosure and analyse the collected samples by gas chromatography mass spectrometry (GC-MS). The VOCs are often either collected passively by solid-phase microextraction (SPME) fibres or alternatively the headspace air is entrained through adsorbent cartridges (Bouvier-Brown et al. 2007; Tholl et al. 2006). The samples can then be either eluted with a solvent, or the entire cartridge can be loaded into the GC-MS and analysed using the technique of thermal desorption. The GC-MS technique is a gold standard in analytical chemistry, but the analysis of a sample takes a long time (approximately half an hour to an hour). In principle, a fast passive sample collection device could sample frequently with subsequent analysis over a longer period, but if the compounds collected are reactive or do not adsorb efficiently this approach may not be sufficient to capture the initiation and evolution of chemical communication signals. Recently, a fast GC-MS technique was demonstrated (Jones et al. 2014) that can reduce the time of analysis by a factor of 2–5. While this is a huge improvement in time resolution, it is not close to real time.

Real-time techniques, which are already widely used in environmental sciences, may help to answer various questions related to interactions occurring over larger distances and in tracking short-term bursts or spiking chemical signals. One common real-time technique that has been used widely in environmental sciences is proton transfer reaction mass spectrometry (PTR-MS) which has the capability to measure at a fast rate (e.g. <1 s time resolution). There are currently two versions of the PTR-MS instrument: (1) a conventional instrument with a quadrupole mass filter (QMS) and (2) an instrument featuring a time-of-flight (TOF) detector. The compound identification is limited to the nominal mass in the case of the instrument with the QMS, but in the case of the TOF version the chemical formulae can be easily identified from the exact mass. Typical instrumental conditions (constant energy field) do not allow for discriminating molecular structures of these chemical formulae. For example, the $C_{10}H_{16}$ ion represents total monoterpenes. In order to separate the mixture of isomers, one would require a combination with other techniques (e.g. GC) or running an instrument in a variable energy mode (variable energy field). This approach would still be challenging for identifying compounds in complex mixtures, but it can discriminate single compounds, for example specific monoterpenes (Misztal et al. 2012). An additional advantage of PTR-MS is that it is highly sensitive to oxygenated molecules (e.g. ketones, aldehydes, acids, esters) which are challenging to measure with commonly used GC columns without specific approaches (e.g. derivatisation of OH groups).

4.3 Ecosystem Fluxes of Volatiles

Due to natural variability in the chemical responses of leaves and plants of the same species, the responses seen at a small scale may not be representative of an ecosystem or even of a big tree consisting of thousands of leaves (Guenther et al. 2012). Responses to light, temperature, stress and the interactions between organisms are commonly non-linear (Reichstein et al. 2014), and thus direct measurements at larger scales can be more representative of an ecosystem than if the individual leaf level responses were scaled up to an entire ecosystem consisting of billions of leaves, flowers and other interacting organisms. Another challenge of measurement at small scales is the possibility of stress introduction or altering the response of the plant due to the measurement setup such as branch enclosures (e.g. Teflon® enclosures alter light scattering, affect CO_2 level, can have an excess of condensing humidity and mechanical damage to plants can easily occur). It seems, therefore, exciting to complement the knowledge obtained from conventional small-scale measurements, with ecosystem-scale measurements that are completely non-invasive and can give a direct representation of the ensemble composition and flux of semiochemicals at a broader scale. This could ultimately lead to a more complete understanding of plant communication and interactions with community members.

Eddy covariance (EC) flux measurements are based on fundamental physics principles and are a direct measure of an ecosystem-scale emission (or deposition) that is routinely used in atmospheric sciences (Bamberger et al. 2011; Ruuskanen et al. 2011; Park et al. 2013b), but is virtually unheard of in studies of chemical signalling in chemical ecology. By just measuring the concentrations of chemicals in the air, questions arise about the origin of a compound and whether it is: (1) emitted from a local source; (2) deposited from a distant source to a local sink or (3) entrained from above the inversion layer (e.g. the nocturnal boundary layer). Measurement of EC fluxes of different compounds can help to address these questions and point to the sources and sinks and their chemical strengths. The chemical vertical flux can be regarded as an exchange rate of molecules between a well-defined area (e.g. a patch of a forest, a flower meadow) and the atmosphere. This exchange is bidirectional with some compounds experiencing emission (positive flux) and deposition (negative flux). A net flux equal to zero could be explained by equal deposition and emission rate or lack of exchange. Closed canopy forests often do not exchange VOCs in a continuous fashion, but in so-called ejections (or bursts) or sweeps (Steiner et al. 2011).

Measurements of both the chemical concentrations and vertical wind speeds at high time resolution enables a flux calculation based on the covariance of these values. The flux can be regarded as emission when the covariance has a positive sign or deposition if the covariance of concentrations and vertical wind speed is negative. An upwind area which represents the flux is called the footprint; it can be precisely calculated and depends in principle on the measurement height, surface roughness and wind speed (e.g. Kormann and Meixner 2001). Looking at larger scales (e.g. footprints of a few hundred metres) will help understanding VOC-mediated communication. From one perspective, it could be considered analogous to listening to the voice of a community consisting not of the response from a single individual, but of all individual groups or species, whose communication could in principle be deconvoluted from net fluxes of specific volatiles. We can imagine a forest under drought, under bark-beetle attack, under wounding stress from hail storms or under heat stress due to an anomaly driven by climate change, and we can look at variations of the individual chemicals (or groups of chemicals) and their magnitudes as well as their prevailing vertical direction (emission, deposition) and horizontal movement (advection).

Eddy covariance can be applied by combining fast measurement of volatiles by PTR-MS (usually 10 Hz, i.e. ten samples per second) with fast vertical wind speed measurements. A tower (or mast) vertically extending several metres above plant canopies is typically used and enables measurements of representative flux from a given footprint, which is an area with a radius many times larger (e.g. 10×) than the measurement height and can be calculated from measured micrometeorological parameters. These types of measurements enable wind sector analysis, where the direction that a specific pheromone is coming from or a sink (a receptor) of another semiochemical can be detected. A relatively small ecosystem footprint of measured flux helps to focus on the sources and sinks of a given chemical within an ecosystem, while at the same time the volatile concentrations are informative of much larger spatial scales depending on the reactivity of the compound. Non-reactive compounds can travel up to hundreds of kilometres (Heil and Ton 2008). These advected compounds, if not emitted or taken up by an ecosystem, may show up as high concentrations, but their flux could be small. In contrast, the compounds emitted by ecosystems (or actively taken up) typically show both high concentrations and fluxes. Some compounds that are actively transported may show low concentration during a day but high flux. This is why both concentrations and fluxes should be measured. Finally, current multivariate and pattern recognition methods such as positive matrix factorization (PMF) or wavelet can potentially extract information about species-specific or process-specific variance in the compounds over an ecosystem. The PMF is an often used tool in atmospheric sciences for elucidating source factors from VOC profiles (e.g. Guha et al. 2015) and could be promising for elucidating signalling in plant communication if all specific semiochemicals were measured and showed a significant source strength. This might seem challenging because volatile-mediated communication is often facilitated by chemicals that occur at concentrations below the average concentration of the most abundant group of VOCs, but by both using high sensitivity TOF instruments and playing down the influence of the most abundant VOCs by data transformation to relative variation, successful results could potentially be obtained. On the other hand, wavelet analysis is a relatively new mathematical tool which has recently been used in various studies for extracting patterns (e.g. in forensics, long-term trends of GHG fluxes and image analysis). Recently, Luo et al. (2013) showed that aphid density can be extracted using wavelets from spectroscopic remote sensing data. Combination of these techniques and volatile dynamics could help, for example, in understanding migration of insects driven by volatiles or tracking effects of stresses on plant–insect interactions.

Stepping up from the ecosystem to the regional scale, BVOC concentrations and fluxes can be measured from an aircraft (Misztal et al. 2014). An example of a compound that plays a crucial role in mediating stress responses is methyl salicylate, which can be measured directly to infer plant stress at the ecosystem scale (Karl et al. 2008). Owing to recent mass spectrometric advances, including the development of PTR-TOF-MS, the actual number of volatiles that can be detected from a single ecosystem is often several hundred and is expected to increase exponentially in the coming years with detection limits expected to reach the lower ppq level (10^{-15}). Looking at the full dynamic composition of ecosystems may help in deciphering the chemical signals of different species as communities (rather than individuals) and in understanding how these interactions change diurnally at the larger scales.

The rest of this chapter presents the principles of real time volatile analysis and eddy covariance measurements and shows example approaches that could be used in studying chemical communication at a larger scale.

4.4 Real-Time Measurements of Plant Volatiles

4.4.1 Proton Transfer Reaction Mass Spectrometer

The proton-transfer-reaction mass spectrometer (PTR-MS) (Hansel et al. 1995; Lindinger et al. 1998) is a fairly recent but enormously useful tool for quantifying VOCs at real time or close to real-time resolution. Due to an ultra-low detection limit (lower part-per-trillion) and a very fast response time (<0.2 s), it has been used in numerous applications since its first use about two decades ago, mainly in environmental sciences, chemistry, biology, medicine and food control. Examples of specific applications include atmospheric composition measurements (Goldstein and Galbally 2007; de Gouw and Warneke 2007), pollution monitoring over cities (Gentner et al. 2012; Shaw et al. 2014), measuring biogenic emissions from vegetated regions (Guenther et al. 1996; Fares et al. 2011), medicine and health (Amann et al. 2004), and in food quality assessment (Biasioli et al. 2011). Some interesting uses of PTR-MS are in measuring BVOC emissions from ornamental trees in cities (Owen et al. 2003) and in odour studies/detection (Hartungen et al. 2004), indoor air quality (Schripp et al. 2014) and it shows promise for application in comprehensive metabolomic profiling (Farneti et al. 2014). Recently, the applicability of PTR-MS has been extended to measurements of semi-volatile and aerosol VOCs (Hellén et al. 2008; Holzinger et al. 2010). Particularly interesting prospects include the real-time measurement of volatiles to elucidate the mechanisms of volatile-mediated interactions, both at fine and larger scales, although so far there have only been a few studies to have looked at these. For example, Schaub et al. (2010) monitored herbivore-induced VOC emissions in the field in real time, while Davison et al. (2008) and Brilli et al. (2012) measured fluxes of cut-induced VOC emissions from grasslands in the field. Real-time PTR-MS measurements have also been used for studying caterpillar-induced volatiles, which can be related to feeding behaviour (e.g. Peñuelas et al. 2007; Laothawornkitkul et al. 2008). Correlation of time-resolved volatile emissions with simultaneous video footage of feeding insects offers an excellent opportunity to match plant responses to precisely monitored damage or stress episodes at a small scale. Recently, volatile-mediated interactions of herbivores and plant root systems were also traced belowground (Danner et al. 2012), offering a potential tool for monitoring interactions where clear visual access is impossible.

A promising future application for real-time PTR-MS measurements relates to monitoring the responses of plants that have received a volatile signal from a damaged neighbour (see Chap. 7). In volatile-mediated plant–plant interactions, one key and frequently reported response in receiver plants is the priming of defences, so that if the receiver plant is subsequently attacked by herbivores or challenged by pathogens it responds more rapidly and strongly than plants that had not received such a signal (Yi et al. 2009; Engelberth et al. 2004; Heil and Kost 2006). Primed defence responses include the emission of induced VOCs in a pattern that is more rapidly elicited and more intense than that of plants not exposed to a damage-related signal. These responses have mostly been monitored with sequential collection of volatile compounds into adsorbent-filled cartridges with subsequent analysis by GC-MS. While this method allows a primed response to be detected, the time resolution is poor. Online real-time volatile measurements offer potentially the best means by which to observe the dynamic pattern of a primed response, which would allow a pinpointing of the precise point that volatile induction begins. This technology has yet to be adopted as a standard for this kind of study, but it is clear that there is the potential to use this tool to progress our knowledge on plant responses to chemical signals and damage events.

4.4.2 Principle of Operation

The technical details of PTR-MS and an operational description are presented in detail by Lindinger et al. (1998). More detailed information on PTR-MS systems can be found in Blake et al. (2009), de Gouw and Warneke (2007) and Ellis and Mayhew (2013).

The chemical principle of operation is the reaction of proton transfer from a protonated water molecule (H_3O^+) to the investigated volatile compound (Reaction R1). Therefore, the recorded mass-to-charge ratio (m/z) of a parent ion will be higher by 1 in relation to the relative molecular mass (RMM) divided by z. In the PTR-MS, the charge of the detected protonated ions is normally always equal to +1, in contrast to common electron ionisation techniques where z can have several values and also a negative sign.

$$H_3O^+ + R \xrightarrow{k} RH^+ + H_2O \tag{R1}$$

Chemical species that can be measured by PTR-MS include all those compounds with proton affinities (PAs) greater than the PA of water, as only for these reactions is proton transfer exoergic and spontaneous ($\Delta G < 0$) at speeds close to the collision rate of reacting ions. The PTR is blind to the most abundant constituents of air for which the PA is higher than that of water (e.g. N_2, O_2, CH_4), which makes the detection of trace VOCs very sensitive and undisturbed by the abundant gases. The list of PAs for common compounds is presented in Table 4.1. In the process of soft ionisation (i.e. relatively low energy of ionisation), the majority of detected ions are molecular ions with little or, in some cases, no fragment ion. This is in contrast to the electron impact (EI) ionisation such as employed in GC-MS systems which result in excessive fragmentation with no or very little of a parent ion consistent with the molecular weight. The degree of fragmentation depends on an important parameter characterising conditions in the reaction chamber (aka drift tube) called the E/N ratio, which is defined as the electrical field density divided by the buffer gas number density. Typically, measurements are conducted with a constant value of E/N, commonly in the range of 110–140 Td (Townsend) ($1\ \text{Td} = 1 \times 10^{-17}\ \text{V cm}^{-2}$).

Table 4.1 List of proton affinities, for example compounds

Compound	Proton affinity (kcal mol^{-1})
Water	165.2
Chemical species with a PA lower than water	
Helium	43
Neon	49
Argon	88
Oxygen	101
Nitrogen	118
Carbon dioxide	129
Methane	130
Chemical species with a PA higher than water	
Formaldehyde	171
Methanol	180
Benzene	180
Acetaldehyde	184
Acetonitrile	186
Toluene	187
Acetone	194
Methacrolein	194
Isoprene	199
Methyl vinyl ketone	200

Data extracted from Ionicon's compilation of proton affinities (detailed lists available online at www.ionicon.com)

At high *E*/*N* ratios, fragmentation is favoured and clustering is minimised, whereas at the low ratios fragmentation is reduced but clustering is enhanced. The latter is manifested by elevated levels of water clusters (*m*/*z* 37, 55, 73, 91, etc.).

In recent times, a time-of-flight (TOF) version of the PTR-MS has been developed (Jordan et al. 2009). The main difference from the standard PTR-MS is the Tofwerk® time-of-flight detector which allows for accurate mass determination and instantaneous acquisition of the entire mass range (e.g. 1.000–1,000.000 Da). Therefore, the concentrations (and fluxes) of hundreds of compounds can now be monitored simultaneously (Park et al. 2013a; Kaser et al. 2012; Ruuskanen et al. 2011). Performance of the quadrupole versus TOF PTR-MS versions has been evaluated by Warneke et al. (2015). The most recent version features TOF with a quadrupole interface for more efficient injection of ions relative to the transfer lens system (PTR-QiTOF-MS) (Sulzer et al. 2014). This provides a remarkable step forward in sensitivity, allowing for detection limits in the range of ppq (10^{-15}). This makes these instruments even more appropriate for monitoring volatile-mediated communication at ecosystem scales because chemical signals can rely on chemicals emitted at extremely low concentrations, for example *cis*-jasmone where the response measured using electroantennography–gas chromatography (EAG–GC) is as strong as that from compounds that are orders of magnitude more abundant (Birkett et al. 2000). EAG could be coupled to the new PTR-TOF-MS systems to understand electrophysiological responses to blends as opposed to single compounds eluted from a GC column. Finally, EAG could be coupled to eddy covariance to inform about the flux of semiochemicals even if the signal of the VOCs is too low to be resolved by mass spectrometry.

4.5 Ecosystem-Scale Measurements of Fluxes of VOCs

The principles of turbulence, advection and transport of chemical species from the ground surface to the atmosphere have been widely described (e.g. Baldocchi 2003; Arya 2001; Lee et al. 2006). Fast response measurements close to real time such as PTR-MS are suitable for use with the direct eddy covariance techniques. There are different techniques appropriate for flux measurements at landscape and regional scales including eddy correlation techniques and the gradient method. These different methods have different advantages and disadvantages; their selection can depend on specific scientific questions and the instrument response time. Here, a brief overview is given.

4.5.1 Eddy Correlation Techniques

Eddy correlation techniques include among others eddy covariance (EC), which is a direct determination of fluxes, and eddy accumulation (EA), which is indirect and can be used with less rapid sensors. These methods are relatively complex requiring a low detection limit and high temporal resolution from gas/particle and wind instrumentation. However, these techniques are appropriate for ecosystem measurements of surface-atmosphere exchange of VOCs including semiochemicals.

Eddy accumulation techniques involve sampling air into two reservoirs depending on the sign of the vertical wind speed. In the true eddy accumulation method developed by Desjardins (1977), the air is sampled proportionally to the vertical wind speed. The largest practical difficulty in realisation of this system has been maintaining constant and fast flow. This difficulty has been overcome in relaxed eddy accumulation (REA), known also as the conditional sampling technique, first presented by Businger and Oncley (1990), and this approach is presented here in more detail. REA is quite widely used in the quantification of organic species emitted over the ecosystem scale (e.g. Nemitz et al. 2001; Graus et al. 2006). Even though this technique is indirect, requiring parameterisations, it has proven useful for quantification of ecosystem fluxes. The principle of the REA method is sampling into two reservoirs (e.g. 0.5 L), one of which is an up-draught reservoir sampling when vertical wind speed (w) is positive, while the other is a down-draught reservoir collecting air parcels travelling downwards ($w < 0$). After an averaging time (typically 30 min), the reservoirs are analysed for the accumulated concentrations of VOCs (c^+ and c^-). In REA, the relaxation technique allows flows into both reservoirs independent of the absolute value of the vertical wind speed. Thus, the vertical flux (Eq. 4.1) is proportional to the concentration difference between c^+ and c^-, the standard deviation of the vertical wind speed σ_w and the dimensionless Businger Oncley parameter, b, which is dependent on the atmospheric stability conditions.

$$F_c = b\sigma_w(c_+ - c_-) \tag{4.1}$$

Another group of flux techniques is based on eddy covariance and relies on correlations of fluctuations in vertical wind speed with those of the components whose flux is to be determined (e.g. temperature, water, CO_2 and VOCs). In other words, at the surface the mean vertical wind speed is assumed to be zero, as the ground is neither a source nor sink for air. Therefore, when a flux of a scalar (e.g. water vapour) is greater than zero, an increase in the positive deviations from the mean value of w are “wetter” than negative portions of the fluctuations, while mean w is still 0. This implies that if there is no correlation between the measured scalar and vertical wind velocity, there is no flux. As the substantial flux in the turbulent planetary boundary layer (PBL) is carried by small eddies, which can only be recorded by either fast sensors (10 Hz) or fast samplers combined with slower sensors, these methods are most suitable for quantification of VOC biosphere–atmosphere exchange, which includes chemical communication signals.

The eddy correlation methods include (continuous) eddy covariance (EC), disjunct eddy covariance (DEC) and virtual disjunct eddy covariance (vDEC), which all are direct methods. More detailed principles of eddy covariance have been described by Moncrieff et al. (1997), Guenther (2002) and Baldocchi (2003). In general, the eddy flux of any scalar can be written as:

$$F_c = \overline{w\rho_c} \tag{4.2}$$

where F_c is the flux density of scalar c, w is the vertical wind velocity, ρ_c is the density (or concentration) of the scalar. The overbar represents the mean of the product over the sampling interval.

The virtual disjunct eddy covariance (vDEC) technique (Karl et al. 2002) is a variant of disjunct eddy covariance relying on a disjunct sampler (Rinne et al. 2001). The vDEC method takes advantage of the conventional PTR-MS as both the fast sampler and analyser.

For the conventional PTR-MS, it is necessary to preselect the ions of interest because the quadrupole detector can measure only one m/z at a time and the cycle needs to be kept short for the flux. The so-called multiple ion detection (MID) mode cycles through 5–20 compounds under study at 0.2 s integration time (dwell time). For example, if 8 compounds are measured at 0.2 s dwell time, the analysis time for one full cycle is ~1.6 s plus a short time (10 ms) for switching from one m/z ratio to another. This example is visualised in Fig. 4.2. In contrast, the TOF-based PTR-MS systems acquire the full spectrum consisting of many hundreds of exact ions in a split second, so the PTR-TOF-MS instruments offer a breakthrough in comprehensive total VOC flux measurements when pre-selection of ions is no longer needed.

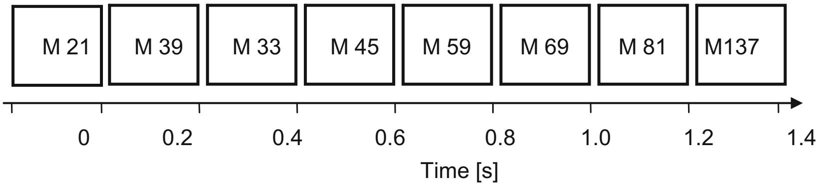

Fig. 4.2 Example of a vDEC duty cycle for 0.2 dwell time of a conventional quadrupole-based PTR-MS. The time-of-flight version measures the whole VOC range at once, not by cycling through the mass-to-charge ratios

In order to calculate the flux, a covariance of instantaneous deviation in vertical wind velocity and the corresponding instantaneous deviation in concentration of a given VOC is calculated, but it needs to take into account the lag time caused by a residence time of the measured compound in the tubing system, relative to wind data received instantaneously (Eq. 4.3)

$$F_c(\tau_{\mathrm{eff}}) = \frac{1}{N}\sum_{i=1}^{N}(w_i - \overline{w}) \times \left(c_{i+\frac{\tau_{\mathrm{eff}}}{\Delta t}} - \overline{c}\right) \tag{4.3}$$

where τ_{eff} is the time lag between the two series $w(t)$ and $c(t)$, and N is the number of samples (e.g. 18,000 in a half hour if measured 10 times per second). Overbars represent time average (e.g. half an hour).

4.5.1.1 Flux Assumption and Principle Corrections

In general, the following assumptions apply during application of EC techniques:

1. Flux is fully turbulent (i.e. most of the vertical flux is carried out by eddies, not by diffusion).
2. Area from which the flux is measured (footprint) is representative for the ecosystem.
3. Terrain is horizontal and uniform.
4. Flux losses with height (divergence) are negligible.
5. Measurements at a point can represent an upwind area.

For high accuracy EC techniques, frequency response and other corrections may be needed (e.g. Lee et al. 2006).

Flux losses associated with signal damping due to residence time in the tubing can be assessed by comparing water vapour fluxes (e.g. derived from the calibrated water vapour ion, *m*/*z* 37 in the PTR-MS), calibrated using a specific humidity sensor and compared with water vapour fluxes from an open path gas analyser.

Flux quality control procedures such as stationarity tests, turbulence criteria testing and other procedures are often essential components in flux processing. While these mathematical procedures might seem a little complex for novice users, they are clearly defined and there are numerous widely available software tools which help processing the data. The quality control procedures and ranking were described, for example, by Foken et al. (2004).

4.5.2 Flux-Gradient Method

The flux-gradient method allows for calculation of fluxes from concentration gradients and relies on measurements of VOCs at two or more different heights in the surface layer (the part of the atmosphere closest to the ground surface—typically it is the lowest 10 % of the atmospheric boundary layer). The flux-gradient method is fairly easy to set up and does not require knowledge of the surface roughness or surface temperature. This method can be suitable when the turbulence is not sufficient for eddy correlation derivation or when the sensor frequency is not sufficiently high for eddy covariance methods. In general, this theory states that in a horizontally homogenous terrain, the mean flow and turbulent characteristics depend only on four independent variables, namely the height, friction velocity, surface heat flux and the buoyancy variable. The distance between the heights should be appropriately chosen [generally the ratio of height at one level to the height of another level (z_1/z_2) should fall between 2 and 4]. The concentration gradients ($\partial C/\partial z$) can be calculated and the flux derived from the following equation:

$$F = -K\frac{\partial C}{\partial z} \tag{4.4}$$

where K is the eddy diffusivity or turbulent exchange coefficient ($m^2\ s^{-1}$). From analogy to Fick's first law of diffusion, the K coefficient needed for the flux calculation can either be obtained by using the concentration of a tracer, e.g. H_2O or CO_2, (modified Bowen-ratio technique) or by surface layer similarity theories. The disadvantages of this method include different footprint characteristics at various heights, measurements at more than one height can sometimes be difficult (e.g. in aircraft measurements) and difficulties in parameterising values of eddy diffusivities in strongly stratified conditions. A more precise derivative of this method is the profile method, or gradient profiling, where the sampling inlet is moved up and down using a winch system (e.g. Karl et al. 2004). This allows for obtaining vertical concentration distribution within and above the forest canopy. The height-integrated concentrations can provide the storage term which is dominant at night (~80 %), while during the day VOCs undergo turbulent exchange between the forest canopy and the atmosphere, and the profile method enables calculating the distribution of sources and sinks using an inverse Lagrangian transport model (e.g. Nemitz et al. 2000). The dynamics of VOC exchange in a forest ecosystem may impact on organism functioning, and during the day the turbulent flow is much more efficient than diffusion for transport of semiochemicals actively from and into the forest (or other ecosystem).

4.6 Future Science Questions to be Addressed at Ecosystem Scale

Future projects should consider using the PTR-MS technique to determine concentration gradients and couple them with techniques used to investigate ecological interactions. One example could be to utilise eddy covariance flux measurements on towers and the measurements of odour plumes (or scentscapes) from aircraft and to relate or overlay that information with the orientation of butterflies or other insects monitored by harmonic radar (Chapman et al. 2011; Ovaskainen et al. 2008). In the same way, spatial VOC data could be overlaid with data on bird movement. The orientation of birds fitted with GPS tracking devices could be mapped relative to the eddy covariance flux measurements to determine whether birds use volatile cues in their natural environment as indicated in earlier studies (see Chap. 9). Measurement of the volatile chemicals eliciting an olfactory stimulus by electroantennography (EAG) should give a response sufficiently fast that we can measure the EC flux relative to the total EAG response of an insect species that characterises an ecosystem. Real-time electrophysiological responses combined with wind direction and speed measurement seems appropriate for tracking the spatial origins of pheromones and other semiochemicals in so-called EAG roses (wind roses). This could be done, for example, from a tower, as demonstrated by Milli et al. (1997) for studying the spatial distribution of pheromones in an apple orchard. Flying drones could also be used to characterise chemical space and record electrophysiological spatial variability. With a combination of comprehensive real-time VOC measurement techniques and insect distribution data, the big picture of plant–plant and plant–animal interactions could be made clearer. It appears that techniques commonly used by different scientific communities might be effectively combined to answer some long standing questions in the field of chemical ecology and plant communication.

4.7 Conclusions

Measurements of volatile chemicals at the ecosystem scale are not commonly used in chemical ecology, but could potentially be very informative for characterising sources and sinks of stress, defence, pollinator attraction, herbivore repelling or other communication signals. Approaches to ecosystem-scale measurements have been refined over the years thanks to the atmospheric sciences community, so the technology exists to make rapid advances in relating these large-scale measurements to ecological interactions. In order to achieve meaningful fluxes of semiochemicals, the detection limits have to be extremely low, otherwise only the most abundant VOCs can be measured, which are more representative of plant metabolic processes and may or may not be relevant to communication processes. However, with the highly sensitive PTR-TOF-MS and PTR-QiTOF-MS, fluxes of hundreds of compounds (Park et al. 2013a) can be measured and the number of detected compounds rises exponentially as sensitivity increases by each order of magnitude. So far, airborne eddy covariance using aircraft has only been demonstrated for the most abundant compounds such as isoprene (Misztal et al. 2014), but when these highly sensitive TOF approaches are eventually used on aircraft, they will enable spatial coverage of and distributions of thousands of compounds. It is therefore very likely that the next 10 years will see breakthroughs in understanding chemical ecology at the ecosystem scale. Including measurements at the larger scale will allow for a holistic embracement of chemical complexities from molecular to regional levels.

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Part II
Ecology of Volatile-Mediated Communication Processes

Chapter 5
Who's Listening to Talking Plants?

Emilio Guerrieri

Abstract In the last few decades, incredible advances have been made in characterising the nature, the origin and the function of plant volatile compounds. These molecules/blends regulate important functions of plant life linked to primary and secondary metabolism. In this chapter, I will focus on the role of these compounds in plant defence against insects (direct and indirect) as determined by constitutive or induced release. I will indicate the possible targets of volatile compounds that become signals in plant–plant and plant–insect communication. I will then conclude with a possible scenario for the exploitation of plant volatiles as a sustainable tool in plant protection against agricultural pests, along with gaps in current knowledge that hamper wider application in the field.

5.1 Introduction

Sessile organisms must develop and reproduce like mobile ones and plants are no exception. This condition poses enormous problems particularly when the time comes to defend because there is no way to escape. However, far from being passive victims, plants have evolved complex and effective strategies to monitor and cope with biotic stresses. Not surprisingly, these strategies are mostly based on the production and release of chemical compounds.

Among the biotic stresses, insects can be considered a major threat to plants, which has generated an endless co-evolutionary process including attack and counter-attack strategies. There is a great deal of literature about plant compounds that are toxic to insects, and agriculture has exploited them in different ways, e.g. in breeding programmes or to produce plant-derived insecticides. This defensive mechanism has been referred to as direct defence because it targets the invading herbivores by reducing their fitness (growth, reproduction), potentially resulting in

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their eventual death. The pioneering work of Price and collaborators (Price et al. 1980) added a new tile to the multifaceted mosaic of plant interactions, the volatile organic compounds (commonly referred to as VOC), which were proposed to help plants defend themselves against herbivores by recruiting natural enemies, a mechanism defined as indirect defence. A trade-off between direct and indirect defences is expected in terms of metabolic costs for the plant (Ballhorn et al. 2008). Since then, the identity, specificity and the metabolic pathways involved in the production (and subsequent release) of these biogenic molecules have been assessed in a number of multitrophic systems.

However, higher trophic levels are not the only targets of plant VOC. Other parts of the releasing plant and neighbouring ones, conspecific or not, could 'receive' these signals, resulting in defensive priming, i.e. a change in the ability to perceive and respond to a biotic stress (Conrath et al. 2006). In this intricate network of interactions, Heil and Karban (2009) suggested there is a substantial difference between communication and eavesdropping, based respectively on the presence or absence of a benefit for the emitter plant. The ecological consequences of this difference are important for the possible exploitation of plant signalling in pest control.

Over 90 % of VOCs released to the atmosphere are produced by plants, converting up to 36 % of the assimilated carbon (Kesselmeier et al. 2002). Their production represents the final steps in complex metabolic pathways related to primary and secondary metabolism, regulating all functions of plant life, including growth, reproduction and plant interactions with the environment, as regulated by abiotic and biotic factors (Maffei et al. 2011). In other words, plants cannot avoid releasing them.

To become signals, these compounds should be 'recognised' by a receiver organism leading to a change in its behaviour/physiology. The relative advantage that could be gained by the emitter (the plant or its parts) and/or the receiver drives natural selection. For example, a volatile compound having a defensive role against insects will enhance the fitness of the releasing individual, favouring its survival compared to the rest of the plant population.

Karban (2015) suggested that signals could bring advantages to the releasing organism. This cannot be entirely applied to plant volatile compounds. Indeed, the very same compound/s could also be favourable or unfavourable for the releasing plant depending on who is 'receiving'. For this reason, I prefer to follow the terminology that defines the role of a volatile compound regulating plant communication on the basis of the possible advantage or disadvantage for one or both partners in the communication (Vet and Dicke 1992). In this view I will use the term kairomones for those compounds bringing a disadvantage to the emitter plant and synomones for those compounds bringing an advantage to both the emitter plant and the receiver organism (be it an insect or another plant). Those compounds that bring an advantage to the receiving organism without apparent advantage/disadvantage for the emitter plant fall into the category of eavesdropping as defined by Heil and Karban (2009).

In the last decade, the study of plant VOC has received a substantial prompt from the more sophisticated tools available for volatile collection and identification. Nonetheless, we are far from reaching the olfactory sensitivity of insects, which explains the frequent discrepancy between collected volatiles and behavioural observations.

A first, crucial distinction should be made between compounds produced by the plant constitutively and associated with what is considered a healthy plant and those induced by biotic (and also abiotic) stresses. Indeed they could target different organisms, subject to different selective pressures. The constitutive release of volatile compounds is influenced by a number of intrinsic and extrinsic factors. Apart from physical parameters including temperature, relative humidity, atmospheric pressure and photoperiod, plant age and phenology play an important role in the composition of volatile blends released constitutively (see Chaps. 1 and 2). For example, flower scent could partially or totally obscure the volatile emission of vegetative parts released by a healthy plant, with consequences for its interactions with non-pollinator organisms.

The literature on induced volatiles is incredibly vast because of the immense number of plant systems investigated, including model ones (see Heil 2014 and references therein). As indicated for constitutive volatiles, the release of induced compounds is influenced by abiotic factors (see Chap. 2) but also by the contemporary presence of multiple attackers (see Chap. 3). In fact, plants are always challenged by diverse stimuli and they selectively respond to them following a priority (Marder 2012, 2013). This plasticity confers a high efficiency to plants in terms of contingent defence (Marder 2012, 2013). Better and quicker responses, resulting in individual and eventually species advantages, are favoured by natural selection. However, more recent findings indicate the existence of a memory in plant response to a stress already experienced that reduces the time and the extent of response (Karban 2015).

The definition of constitutive and induced volatiles has been made more complicated by recent advances in plant symbioses. Indeed, it has been demonstrated how root symbionts, such as mycorrhizal fungi or antagonistic fungi, could alter the release of plant volatile compounds (Guerrieri et al. 2004; Battaglia et al. 2013). Considering that mycorrhizal symbiosis is reported for nearly 80 % of known plant species (Smith and Read 1997), what has been considered so far a constitutive release could be in fact induced by belowground symbiosis. However, those compounds whose release is not induced by a biotic stress I will refer to as constitutive.

Because VOCs are involved in direct and indirect mechanisms of plant defence (e.g. Sasso et al. 2007; Digilio et al. 2010, 2012), we can hypothesise that the metabolic costs of induced volatiles are lower with respect to those of constitutive ones (Stamp 2003). However, it has been observed that allocation costs of plant defence are usually calculated in terms of C and N and this could possibly reduce our complete understanding of the phenomenon (Karban 2011).

In this chapter, I will focus on aboveground multitrophic interactions among plants, pests and natural enemies as regulated by the emission of VOC, leaving plant–pollinator interactions (Chap. 11) and plant belowground interactions (Chap. 8) to more expert authors. I will try to outline when volatile compounds become ‘signals’ and how they could be exploited to enhance the control of insect pests in agriculture. I will conclude with future scenarios and challenges for research on plant VOC.

5.2 Who Targets Plant Volatile Signals?

5.2.1 Plant Insect Interactions

5.2.1.1 Plant–Herbivore Interactions

The blend of volatile compounds characterising each plant species is exploited by insect herbivores to unambiguously locate their hosts for feeding and oviposition (Bruce and Pickett 2011) (Fig. 5.1). Recognition of a host plant using these olfactory signals could occur by using either species-specific compounds or specific ratios of ubiquitous compounds. In some cases, a crucial role is played by single compounds. This is particularly evident for plant families such as Brassicaceae where the role of constitutive glucosinolates as powerful contact elicitors of feeding and oviposition in specialist herbivores has been documented both above- and belowground (Louda and Mole 1991; Bruce 2013). Among the breakdown products of these compounds, there are volatile isothiocyanates that act as powerful long-range attractants for specialist insect herbivores feeding on Brassicaceae (Wittstock et al. 2003; Bruce 2013). Glucosinolates and their metabolic derivatives could be considered true constitutive compounds because Brassicaceae are among the few

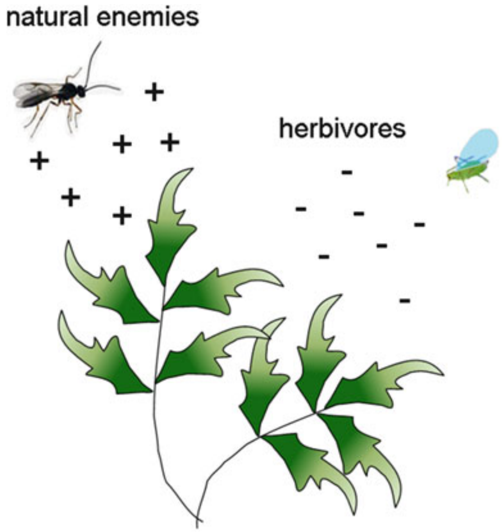

Fig. 5.1 Targets of constitutive release of plant volatiles interpreted from the perspective of the emitter plant—*plus symbols* indicate a positive attraction; *minus symbols* indicate a negative attraction

non-mycorrhizal plant families (Smith and Read 1997). Sometimes, attraction towards these volatiles is not innate but could be gained by specialist insects after a feeding experience, as is the case for *Athalia rosae* attacking *Brassica* plants (Barker et al. 2006).

However, in most plant systems, it is a specific blend, as constituted by specific ratios of specific compounds, which regulates plant attractiveness to a herbivore (Bruce and Pickett 2011). The perception of a blend in place of single compounds offers higher plasticity and reliability. This applies to those ubiquitous compounds that cannot be related unequivocally to any plant species (or family) and cannot be 'perceived' as signals if isolated from the original blend (Bruce and Pickett 2011). Even seeds are sources of volatile blends, reliably guiding the foraging behaviour of many phytophagous insects. For example, adults of the grain beetle *Sitophilus zeamais* Motschulsky are selectively attracted by a blend of hexanal, (*E*)-2-heptenal and octanal towards maize seeds (Ukeh et al. 2012). The attraction/recognition–single compound/blend models have also been reported for tree systems. The terpene α-pinene, released by pine trees, is by itself a powerful kairomone in the attraction of the pine shoot beetle (Zumr 1989). Conversely, the constitutive emission of a blend of monoterpenes, mainly (*E*)-β-ocimene and linalool, by the young leaves of poplar is responsible for attraction of the phytophagous beetle *Chrysomela populi* L. (Brilli et al 2009). Feeding by adult beetles enhances the release of the attractive blend (induced release) and in turn the rate of herbivore attack (Brilli et al. 2009).

There is an evident negative pressure on those plant individuals that are identified more quickly as a host in a population by an insect herbivore (Labandeira 2013). Similarly, those herbivorous insects less able to find their substrate will be outcompeted by more 'sensitive' ones (Labandeira 2013).

More rarely, constitutive volatile emissions can be extremely efficient in protecting the plant from its herbivores, as is the case for *Solanum berthaultii* (Hawkes). One of the main components in the volatile emission of the glandular trichomes of this wild potato is (*E*)-β-farnesene, a major constituent of aphid alarm pheromone. Foraging aphids are so efficiently repelled by this compound that *S. berthaultii* is virtually immune to these sap feeders (Gibson and Pickett 1983). The constitutive release of defensive volatiles, though desirable in cultivated plants, requires high metabolic costs that could hamper the quantity and the quality of the yield. For this reason, cultivated varieties have virtually lost the ability to repel their herbivore invaders, having been exclusively selected for production quality and quantity (de Lange et al. 2014). However, there are ways to recover these features through selective breeding or transgenesis, if wild ancestors and congeneric species are available, as is the case for tomato and maize (Bleeker et al. 2012).

Compared to constitutive defences, those defences mounted only when necessary (induced defences) are more affordable to plants in terms of metabolic costs and, in some cases, can be extremely effective (reviewed by Hare 2011). Because of the impossibility of escape, plants have evolved sophisticated mechanisms to detect and respond to a wide range of attackers including insect herbivores. This is particularly surprising for sap-feeding pests that cause very little mechanical damage to plant tissues. For example, in response to aphid attack, tomato plants release higher amounts of several compounds including methyl salicylate and (*Z*)-3-hexen-1-ol (Sasso et al. 2007). These volatiles are involved in tomato direct defence against aphids by altering their settling behaviour and by reducing their fertility in the early phases of attack (Digilio et al. 2012) (Fig. 5.2). In the intricate plant–aphid interactions, viruses also have an important role, regulating the extent of transmission and diffusion with dramatic consequences for agricultural crops. Volatiles induced by potato leaf-roll virus (PLRV) make plants more attractive to winged (but also apterous) *Myzus persicae* (Sulzer) (Ngumbi et al. 2007; Werner et al. 2009). These differences appeared to be linked more to the ratio among compounds than to the increased release of specific ones (Ngumbi et al. 2007). The lethal association between aphid vectors and viruses reaches its highest efficiency in cucumber plants infected by the Cucumber Mosaic Virus (CMV). Indeed, not only are these plants more attractive for the aphids *M. persicae* and *Aphis gossypii* Glover, but they are also less suitable for insect growth and reproduction, inducing a higher rate of abandonments, leading to quicker rates of virus transmission in the plant population (Mauck et al. 2010).

Interaction between herbivore-induced volatiles and subsequent herbivore attack has been documented for chewing insects too. Volatiles released by tobacco plants fed on by *Heliothis virescens* larvae were repellent to conspecific ovipositing females (De Moraes et al. 2001). The release of the key compounds involved in these interactions is concentrated in the nocturnal hours and this is coherent with the

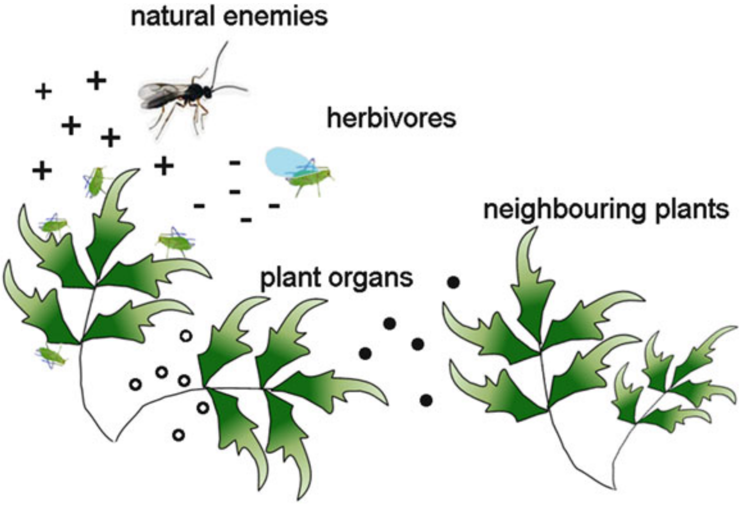

Fig. 5.2 Targets of induced release of plant volatiles interpreted from the perspective of the emitter plant—*plus symbols* indicate a positive attraction; *minus symbols* indicate a negative attraction; *multiplication symbols* indicate a positive repellence; *filled circle symbols* indicate a positive within-plant communication; *open circle symbols* indicate a neutral plant–plant communication

habit of the adult insect studied (De Moraes et al. 2001). Similarly, tobacco plants attacked by *H. virescens* were repellent towards western flower thrips *Frankliniella occidentalis,* and this is due to the increased level of nicotine induced by caterpillar feeding (Delphia et al. 2007). Most surprisingly, volatiles induced by chewing caterpillars are attractive to conspecific larvae in a number of herbaceous and tree systems, even though the insect species studied are not known to be gregarious. For example, *Spodoptera littoralis* and *Cydia pomonella* caterpillars were more attracted to volatiles released by host plants attacked by conspecifics than to uninfested plants (Von Mérey et al. 2013; Landolt et al. 2000). Whereas the repellence of ovipositing females from infested plant hosts can be easily explained as a species mechanism that reduces fratricidal competition and enhances the survival of newborns, this is not particularly true for larval attraction towards larvae-infested hosts. One hypothesis is that the blend of volatiles released by infested plants includes compounds that are readily associated with the host plant (Von Mérey et al 2013). The enhanced response recorded for experienced larvae seems to validate this theory (Von Mérey et al 2013).

In the case of attack by multiple herbivores, induced volatiles warn later arriving herbivores that the substrate is already colonised, resulting in repellence. For example, maize volatile blends resembling those induced by chewing larvae were highly repellent to the winged forms of the aphid *Rhopalosiphum maidis* (Fitch) in semi-field tests (Bernasconi et al. 1998). However, in a few cases, herbivore-induced volatiles have been shown to be used by later arriving herbivores to locate their host plant. Silencing the genes responsible for the release of volatiles by *Nicotiana attenuata* chewed by *Manduca sexta* resulted in reduced attraction of the predatory bug *Geocoris pallens*, but also in reduced attack by the flea beetle *Epitrix hirtipennis* in field tests (Halitschke et al. 2008). It is uncertain whether searching for a plant already colonised by other herbivores represents a successful strategy for a herbivore. Indeed, plant defences elicited by a first invader could be effective against the following one or, conversely, a second herbivore could find a plant that is not capable of reacting. The timing of the following attacks and their identity, in terms of damage, regulates the final outcome of these interactions.

Along with feeding, insect oviposition has been indicated as a powerful elicitor of plant volatiles with specific effects on subsequent herbivore attraction and attack (reviewed by Hilker and Fatouros 2015). The species specificity of these interactions is sometimes surprising. For example, gravid females of the large cabbage white *Pieris brassicae* are repelled by volatiles released by *Brassica nigra* plants induced by previous oviposition by a conspecific female. Conversely, no effect was noted in the case of a previous oviposition by the generalist *Mamestra brassicae* (Fatouros et al. 2015). More recent studies have focused on the role of root symbionts in the induction of plant defence. The arbuscular mycorrhizal symbiosis has proved to have a positive effect on the attractiveness of *Vicia faba* L. towards the aphid *Acyrthosiphon pisum* (Harris) due to a significant reduction in the release of sesquiterpenes (*E*)-caryophyllene and (*E*)-β-farnesene (Babikova et al. 2014a). The positive effect of mycorrhizal colonisation on aphid attraction was correlated with the extent of root colonisation but not with plant nutrition measured as N and P content in the leaves and plant biomass (Babikova et al 2014b). As expected, in this system plant volatiles induced by aphid feeding were repellent to conspecific aphids (Babikova et al 2014a).

Behind these plant responses to herbivore attack, there is the activation of specific metabolic pathways (Walling 2000) with frequent crosstalk between them (Bostock 2005; and references therein).

5.2.1.2 Plant–Natural Enemy Interactions

In the behavioural steps followed by a natural enemy, be it a predator or a parasitoid, the location of the feeding site of the victim (host habitat location) plays a crucial role (Vinson 1981). Indeed, in a complex environment even simplified as an agricultural crop, natural enemies must progressively reduce the searching area by following cues of increasing specificity. In this view, the same constitutive plant volatiles, acting as kairomones towards herbivore insects in their search for a host, could be used by natural enemies as a first effective cue to locate their prey or hosts and thus act as synomones too. The interaction between the plant and the natural enemies of its herbivores has been termed 'indirect defence' to stress that the compounds involved have no direct impact on herbivore populations that are in fact reduced by the intervention of antagonists.

Plant volatiles are abundant in every environment; thus, they can be easily detected even though those released constitutively do not reliably indicate the presence of natural enemies' targets (Vet and Dicke 1992). In the vast literature on plant–natural enemy interactions, uninfested control plants are reported to exhibit some attractiveness to natural enemies (Fig. 5.1). The extent of this attractiveness varies with plant species and variety and in some cases can be surprisingly high. For example, whereas in commercial varieties of tomato (e.g. Better Boy), attractiveness of uninfested plants to an aphid parasitoid (*Aphidius ervi*) reached about 20–30 % of females tested, in ecotypes locally selected for their resistance to aphids it was as high as 60 % (Corrado et al. 2007; Digilio et al. 2010). The consequences of this level of constitutive attractiveness are easily imagined: virtually no population of aphids could develop on these ecotypes, which is also due to the presence of toxic compounds that directly affect aphid development and reproduction (Digilio et al. 2010). As expected, the same aphid parasitoid species was differentially attracted by different cultivars of broad bean plants in wind tunnel tests (Guerrieri et al. 1993; Du et al. 1996).

The use of constitutive volatiles in foraging behaviour seems not to be the behavioural strategy followed by parasitoids 'specialised' on Brassicaceae. For example, *Diaeretiella rapae*, a generalist parasitoid in terms of aphid species attacked, but almost a specialist on Brassicaceae, was not selectively attracted towards the odour of uninfested brassica even when offered clean air as an alternative (Blande et al. 2007). To my knowledge, there is no report to date of constitutive volatile compounds released by trees having an impact on the attraction of natural enemies of their insect pests.

Far more reliable, in terms of information about the effective presence of their prey/host, are volatiles released in response to herbivore attack, generally referred to as herbivore-induced plant volatiles (HIPVs) (reviewed by Heil 2014) (Fig. 5.2). Indeed, for the natural enemies of insect herbivores, these volatiles represent the best trade-off between reliability and detectability (Vet and Dicke 1992). Previous experience can either increase the foraging efficiency of natural enemies (Turlings et al. 1993) or significantly alter their behaviour (Guerrieri et al. 1997). As mentioned above, the induction can be the result of either herbivore feeding and/or oviposition. The reliability of HIPV for the recruitment of natural enemies of insect pests has prompted numerous investigations in several plant–pest–parasitoid systems. The model plants *Arabidopsis thaliana*, *Nicotiana attenuata* and tomato are among the most studied because of the possible application of results for the sustainable control of agricultural pests. However, consistent differences have been reported in the timing of induction and in the subsequent release of induced volatiles following differences in the feeding and ovipositing habits of the herbivore species considered. For example, induction of volatile emissions targeting natural enemies in maize plants occurs during a few hours following caterpillar attack, caused by a combination of mechanical damage (chewing) and chemical elicitation (saliva) (e.g. Alborn et al 1997). Conversely, about 3 days and a consistent aphid population are needed to make a plant attractive to aphid parasitoids (e.g. Guerrieri et al. 1999).

In most cases, the release of HIPV has been reported to be systemic. In other words, chemical signals running through the vascular systems of infested plants extend the emission of these biogenic compounds to undamaged parts of the plant. For example, the apex of plants attacked basally by insect herbivores, either chewers or sap feeders, resulted in the entire plant becoming attractive to the relevant parasitoids (e.g. Turlings and Tumlinson 1992; Guerrieri et al. 1999). The elicitor responsible for the systemic response of tomato plants towards chewing herbivores has been isolated, characterised and named systemin (Bergey et al. 1996). It has been debated whether the attraction of natural enemies towards herbivore-induced volatiles is due to the presence of specific 'compounds' or 'blends' (Bruce and Pickett 2011). While the release of terpenes from the octadecanoid pathway in response to chewer attack can be extremely variable in terms of composition and ratio, some homogeneity has been observed in the volatile composition following aphid attack. As a result of activation of the salicylic acid pathway, methyl salicylate has been found to be a major component in the emissions of aphid-infested plants (e.g. Zhu and Park 2005; Sasso et al. 2007). This compound, though included in different blends released by different plant–aphid systems, appears to be key to the foraging behaviour of aphid parasitoids and predators (Zhu and Park 2005; Sasso et al. 2007, 2009). Indeed, it is detected by the antennae of the aphid parasitoid *Aphidius ervi* at concentrations as low as 0.01 mg/ml (Sasso et al. 2009).

It took almost 20 years to discover that herbivore oviposition could be another powerful elicitor of volatile emissions that are exploited by natural enemies to locate their herbivore victims (Hilker and Fatouros 2015). In herbaceous and arboreous systems, plants are induced not only by tissue damage but also by the ‘glue’ used to fix the eggs to the substrate. For example, volatiles released by the twigs of *Pinus sylvestris* heavily loaded with egg masses of the pine sawfly *Diprion pini* were attractive to the eulophid egg parasitoid *Closterocerus ruforum* (Hilker et al. 2002). Similarly, oviposition by the common green stink bug *Nezara viridula* on *Vicia faba* resulted in a significantly higher attractiveness towards the egg parasitoid *Trissolcus basalis* (Colazza et al. 2004). The ecological advantage for a plant recruiting the egg parasitoids of its herbivores is evident: the defence starts before possible damage is inflicted and the invading herbivore is stopped at its earliest stage of development. The infinitesimal dimension of these egg parasitoids so efficiently responding to ovipositing cues in a complex environment such as a wood or an agricultural crop indicates how powerful is the ecological selection operated by HIPVs.

However, insect parasitoids are not immune from being themselves attacked, and indeed, they represent a foraging target for higher trophic levels of the food chain. Secondary parasitoids, also termed hyperparasitoids, exploit herbivore-induced volatiles to locate their victims, which can be considered as a host habitat location (see Chap. 9). For example, females of the hyperparasitoid *Lysibia nana* positively responded to volatiles from plants damaged by *Pieris rapae* larvae irrespective of whether they were unparasitized or parasitized by either *Cotesia rubecula* or *C. glomerata* (Poelman et al. 2012). As a consequence, the same compounds (blends) acting as synomones in the interaction between plant and parasitoid become kairomones in the interaction between plant and hyperparasitoid. Although it is far beyond the aim of this chapter to be exhaustive on the induction of plant volatiles, it is important to mention that global climatic changes affect the release of constitutive and induced volatiles with unpredictable consequences on the higher levels of the trophic chain (see for example Peñuelas and Staudt 2009; Chap. 2).

5.2.2 Plant–Plant Interactions

Only 3 years after the pivotal paper of Price and collaborators on tritrophic interactions mediated by plant volatiles, Rhoades (1983) reported that the presence of herbivore-infested willow trees in the vicinity of uninfested ones rendered the latter more resistant towards the herbivore. This can be rightly considered as the beginning of investigations on volatile-mediated communication between plants. However, plant volatiles, particularly those induced by biotic stress, can reach and stimulate other parts of the same plant, referred to as ‘within-plant communication’ or neighbouring ones, referred to as ‘plant-to-plant communication’ (Fig. 5.2). Within the latter interaction, it is possible to further distinguish between intraspecific (plant individuals belonging to the same species) and interspecific communication (plant individuals belonging to different species).

5.2.2.1 Within-Plant Communication

The physiological barriers that prevent the diffusion of systemic signals within a plant under herbivore attack could be effectively overcome by volatile compounds. The efficiency of this type of communication has been reported for herbaceous and arboreous plants. For example, the production of extra floral nectar by undamaged leaves of lima bean plants is induced by volatiles released by neighbouring leaves chewed by beetles (Heil and Silva Bueno 2007). Similarly, in poplar trees, volatile messages from infested leaves 'alert' undamaged neighbouring leaves, overcoming the absence of direct vascular connections (Frost et al. 2007). Compared to communication via vascular channels, which do not effectively and uniformly link all parts of a plant, volatile communication among different parts of the same plant is characterised by a higher speed and could be a widespread phenomenon regulating plant defence (Orians 2005). On the other hand, the release of volatile compounds into the air exposes the emitter to the risk of being 'perceived' and, as mentioned above, this does not always produce a benefit (Karban 2015). The volatile esters of salicylic and jasmonic acids, representing the final step in the activation of homonymous metabolic pathways of herbivore and pathogen defence, have both been identified as common intra-plant inducers of defensive responses (e.g. Shulaev et al. 1997).

5.2.2.2 Plant-to-Plant Communication

The induction of conspecifics through volatile communication has been assessed in several systems both herbaceous and arboreous. For example, feeding activity of the leaf beetle *Agelastica alni* is negatively affected by defoliation (Tscharntke et al. 2001), and the level of induced resistance is inversely proportional to the distance (up to 10.6 m away from defoliated plants). More recent studies have highlighted that interplant communication by volatile compounds, particularly over a distance, acts as a primer of defence more than an inducer, with consistent differences in the expected expression of defensive genes. This correlates to volatile concentration and in turn to the distance between communicating plants (Heil and Ton 2009). The accumulation of defensive proteins that make the plant more prepared for future attacks and the possible removal of repressors of defensive genes have been indicated as possible mechanisms of priming/conditioning induced by plant exposure to volatile compounds (Karban 2015). However, it appears that genetic similarity results in better communication between congeneric plants as demonstrated in sagebrush (Karban et al. 2014). In fact, volatile interactions between different cultivars of undamaged barley can induce a resistance response measured as reduced aphid acceptance, but this also happens after barley is exposed to thistle even though the timing of these interactions is critical (Glinwood et al. 2007).

Interspecific communication between plants as a mechanism of defence induction is a relatively recent discovery (Shulaev et al. 1997; Arimura et al. 2000). A number of volatile compounds that appear to be commonly related to herbivore attack, regardless of plant species, act as interspecific signals inducing a defensive response. For example, methyl salicylate and methyl jasmonate, whose release dramatically increases after herbivore attack in several plant systems, are both powerful inducers of plant defence when received by uninfested plants (Shulaev et al. 1997; Tscharntke et al. 2001; Tamogami et al. 2008). Studies involving the model plant *Nicotiana attenuata* revealed the intricate conversion of volatile signals coming from neighbouring plants into defensive responses and the possible differences emerging from laboratory and field tests. The blend of VOC released by clipped sagebrush was indeed responsible for defensive priming in neighbouring native tobacco plants, but methyl jasmonate, found to be an active elicitor in laboratory tests, was not produced in sufficient amounts in the field to induce a defensive response in receiving plants (Kessler et al. 2006).

Among volatile compounds acting as defensive primers in neighbouring heterospecific plants, β-ocimene and (*Z*)-jasmone have been thoroughly studied (e.g. Arimura et al. 2000; Birkett et al. 2000). The role of β-ocimene has been assessed in lima bean plants by using transgenic tobacco overexpressing the ocimene synthase (Arimura et al. 2000). More recently, this compound was found to be particularly active in eliciting both a direct and indirect defensive response towards aphids in receiving tomato plants (Cascone et al. 2015). There are questions raised about the ecological significance and benefit of interplant communication. Given the unavoidability of volatile release in response to herbivore damage, there is a common line of thought that considers these compounds to act more as intra-plant than inter-plant messengers of danger (Heil 2014). If this is the case, we should expect intra-plant signals to be more specific. However, the possible benefits in terms of priming induced in neighbouring heterospecific receiver plants could result in an evolutionary driving force towards plants more able to eavesdrop on signals and then more ready to be primed against invaders.

5.3 How to Exploit Plant Signals for Pest Control in Agriculture?

In the last two decades, there has been a bottom-up request for sustainable methods of pest control, and the use of volatile compounds or plant elicitors can be rightly considered two such methods. Since the earliest evidence of the role of volatile compounds in either repelling herbivores (direct defence) or attracting their natural enemies (indirect defence), there has been an increasing expectation about their use as pest control tools (Sobhy et al. 2014). However, so far, the only success at large scale obtained with behavioural manipulation mediated by plant volatiles has been the so-called push and pull strategy realised in Africa for the control of maize and sorghum stemborers (reviewed by Cook et al. 2007). This technique is based on a simple model where pest insects are pulled away from the cultivated crop by using repellent plants and pushed onto a non-commercial attractive one, while the opposite applies to their natural enemies. The interactions are all mediated by constitutive and induced plant volatiles resulting in sustainable control of insect pests without the use of synthetic pesticides. Alteration of insect behaviour induced by specific volatile compounds/blends could be similarly obtained through either the use of specific elicitors or transgenic plants.

5.3.1 Plant Elicitors

A number of compounds act as elicitors of plant defence, sometimes involving the release of volatile compounds having an effect on the control of pests. For example, treating maize plants with BTH ((1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester), a mimic of salicylic acid, resulted in a higher attractiveness of *Microplitis rufiventris*, a caterpillar parasitoid (Rostás and Turlings 2008). This result, somewhat unexpected considering the reported antagonism between the metabolic pathways of salicylic and jasmonic acid (Thaler et al. 2002), was confirmed by subsequent tests including another elicitor, laminarin, and explained by a subtractive hypothesis where the suppression of some volatiles not directly involved in parasitoid attraction results in a higher attractiveness (Sobhy et al. 2012).

Methyl salicylate, found in the volatile blend released by several plant species in response to aphid and pathogen attack (see Sect. 2.1), is regarded as an elicitor of plant direct and indirect defences in tomato plants (e.g. Digilio et al. 2012). This compound, alone or in combination with others including methyl jasmonate (James 2005), has been used to recruit beneficial insects into different agricultural systems including hop and vineyard (James and Price 2004; James 2005). More recently, within the framework of the sustainable control of insect pests, methyl salicylate has been used as a lure to develop phenology models for a chrysopid predator (Jones et al. 2015). Both methyl salicylate and methyl jasmonate alter the settling behaviour of the aphid *Rhopalosiphum padi* on barley, confirming the higher complexity of plant responses to sap feeders compared to chewers (Glinwood et al. 2007).

Increased knowledge about the effect of volatile compounds as elicitors of plant direct and indirect defences, together with technical advances in the design of dispensers of volatile compounds, represents a concrete possibility for field application (Uefune et al. 2012). Along with its analogue methyl jasmonate, (*Z*)-jasmone, a component of floral volatiles also released by cotton plants infested by caterpillars (Loughrin et al. 1995; Paré and Tumlinson 1997), has been shown to be a powerful inducer of plant defences against aphids in a number of plant systems (Birkett et al. 2000; Pickett et al. 2007). However, consistent differences were noted in the elicitation of plant direct defences with respect to specialist and generalist aphids attacking the model plant *Arabidopsis* (Bruce et al. 2008).

5.3.2 GM Plants

The release of induced compounds could be selectively obtained by enhancing the expression of relevant genes in genetically modified plants. For example, (*E*)-β-ocimene has been indicated as a regulator of gene transcription and a primer of indirect defences (Muroi et al. 2011). Transgenic tobacco overexpressing the ocimene synthase releases a volatile blend enriched in ocimene, thus acting as a living inducer of plant defence. Lima bean and tomato plants neighbouring these transgenic plants were more protected against mites and aphids, respectively (Muroi et al. 2011; Cascone et al. 2015). In a possible future scenario, intercropping and border cropping in a push and pull strategy could involve transgenic plants releasing volatile compounds and blends specific for plant, pests and natural enemies, thus avoiding the direct transformation of productive varieties. A first step towards this application has been recently tested in the field using transgenic plants releasing (*E*)-β-farnesene, even though the first results were not encouraging (Bruce et al. 2015).

5.4 Gaps in Knowledge and Future Challenges

In the latest more comprehensive reviews on plant volatiles, a number of gaps in knowledge and future challenges have been indicated (Dicke and Baldwin 2010; Hare 2011; Heil 2014). However, many of the indications are still to be followed by research groups around the world. If we really want to use plant volatiles as a sustainable tool to control insect pests, then large field tests, still lacking, are urgently needed. There is a natural reluctance towards field tests by researchers because of the immense, sometimes unmanageable, number of variables but mostly because they are time-consuming. Nonetheless, these tests could give a precise indication of whether the production of HIPVs is really an evolutionary trait shaped by selection or not (Hare 2011). It is indeed possible that the main target of these compounds is the plant itself, to overcome the constraints of vascular communication (Heil and Ton 2009). To unravel this dilemma, a solid demonstration of the plant fitness benefit linked to the emission of volatiles and the attractiveness of natural enemies of herbivores is still needed.

However, particularly for those compounds that have been indicated as released in response to ‘specific’ herbivores, semi-field and field tests with transgenic plants could prompt their future use in a push and pull approach to pest management. In this respect, it appears important to direct future research on plant volatiles towards crop plants in place of model ones. Very few studies have highlighted the circadian release of volatile compounds (e.g. De Moraes et al. 2001, Glinwood et al. 2007) even though in some cases the nocturnal phase is even more important than the diurnal one. In fact most, if not all, laboratory studies with plant pests and natural enemies have been run in daylight conditions, which is not necessarily most relevant for the species considered.

The sophisticated mechanisms underlying plant perception and translation of volatile compounds are still to be unravelled and a great interdisciplinary effort should be made in collaboration with plant physiologists in search of the plant 'nose' (Heil 2014). This goes together with deciphering the concentration at which single or multiple compounds become reliable signals for receiving organisms, be they plants or insects, with immediate critical consequences for field application.

In this chapter, I have focused on aboveground scenarios, but belowground there is still a world to be explored and exploited (see Chap. 8). Here, it is important to mention that the impact of root symbionts on plant volatile release is another variable to be considered in the study of multitrophic interactions, particularly for combined defence against multiple biotic stresses (e.g. Guerrieri et al. 2004; Battaglia et al. 2013).

5.5 Conclusions

The study of plant–insect interactions is a fascinating one, particularly when considering the foraging behaviour of entomophagous insects. There is a large body of evidence that their search for a host (or prey) cannot be random and that volatiles are key signals to be followed. In fact, we still do not know how many plant pests are kept naturally under control by natural enemies in multitrophic systems, possibly regulated and shaped by plant volatiles. The great variability connected with the release of volatile compounds from a plant does not have to discourage investigations, because technical advances in the detection and characterisation of these compounds could give substantial help. In fact, it is unquestionable that plant volatile compounds represent an immense resource to be used as a tool to enhance the biological control of pests that still devastate our agricultural production.

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Chapter 6
Ecological Role of Odour Diversity

Torsten Meiners

Abstract Multitrophic interactions between plants and arthropods of different trophic levels take place in heterogeneous and complex environments, formed primarily by both host and non-host plants. High plant diversity and non-host plants and their interaction with host plants, other trophic levels and abiotic factors may form a diverse odour bouquet that arthropods have to cope with when foraging for food, hosts, prey, conspecifics or mating partners. This chapter focuses on the ecology of the generation of odour diversity and—vice versa—its effect on plants, herbivores and their natural enemies. Future research will need novel experimental approaches to identify the patterns and processes involved in these intricately unique interactions.

6.1 Introduction

Arthropods orientate primarily using olfaction when searching for food, prey, conspecifics or mating partners (reviewed by Bruce et al. 2005; Godfray 1994; Visser 1986). In their natural habitats, they are faced with volatile compounds mainly originating from non-host plants. Plant diversity is known to affect the host location behaviour of arthropods in the field (Bukovinszky et al. 2007; Petermann et al. 2010; Randlkofer et al. 2010; Root 1973; Unsicker et al. 2006) and in laboratory setups (reviewed by Randlkofer et al. 2010). A high plant or genetic diversity of the surrounding vegetation may generate a diverse odour bouquet that arthropods have to cope with when navigating within the habitat (Beyaert and Hilker 2014; Finch and Collier 2000; Ninkovic et al. 2011; Perfecto and Vet 2003; Randlkofer et al. 2007, 2010). However, the relationship between the plant diversity and the resulting vegetation stand odour diversity has received little

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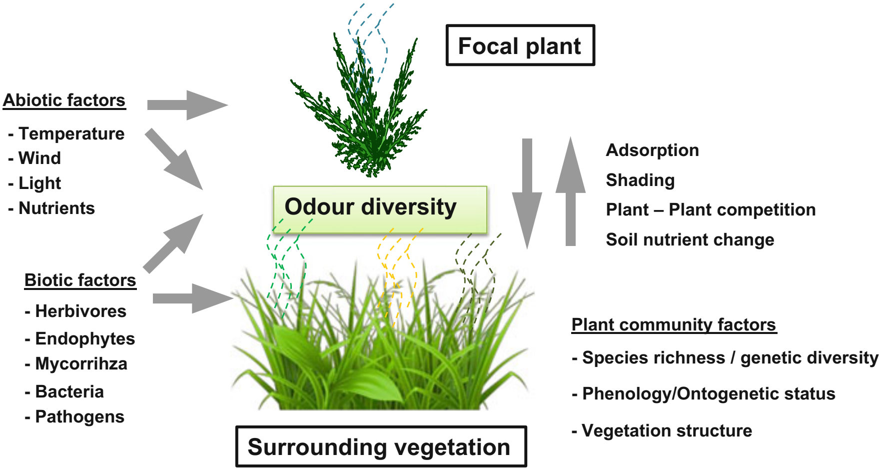

Fig. 6.1 Effects of abiotic and biotic factors on the generation of odour diversity of the vegetation surrounding a focal plant

attention (Iason et al. 2005), as have the responses of plants, herbivores, predators and parasitoids to odour diversity. Including chemical diversity in biodiversity research is an emerging issue in ecosystem function research (Hilker 2014; Meiners 2015; Randlkofer et al. 2010). Vegetation stand odour diversity can modify interactions between plants, herbivorous insects, their natural enemies and higher trophic levels in multiple ways. Plant species diversity strongly determines the vegetation complexity and thus plant chemical and plant structural complexity (Randlkofer et al. 2010). Plant structures affect microclimatic conditions, which in turn influence the emission and dispersion of plant volatiles (e.g. odour diversity). Plant volatile odour diversity may significantly affect neighbouring plants and the orientation of herbivorous and carnivorous arthropods and thus may influence plant–plant interactions, foraging and mating success of arthropods and, finally, community composition. This chapter emphasises an ecological view on the generation of odour diversity of vegetation volatiles (Fig. 6.1) and its effect on plants and arthropods from different trophic levels (Table 6.1).

6.2 Generation of Odour Diversity

6.2.1 Species-Rich Plant Communities

Different plants release a huge variety of volatiles that form the odour diversity of a given vegetation stand. Vegetation stand odour can show a high variability since the biochemistry of plant components is characterised by an enormous diversity and variability of compounds (Hadacek 2002; Ober 2005). From the 200,000 secondary

Table 6.1 Responses of focal plants, herbivores and parasitoids or predators towards enhanced odour diversity

Responses to odour diversity (OD)	Direction of response to OD	Suggested ecological effect	Target of responder	Reference
Focal plant responses				
Biomass allocation	Enhanced	Avoid competition	Neighbouring plants	Ninkovic (2003)
Secondary (e.g. allelopathic) compound production	Enhanced	Avoid competition	Neighbouring plants	Suggested in Wäschke et al. (2015)
Secondary compound production	Reduced	Cost avoidance	Herbivores	Not shown for OD yet, shown for neighbour identity by Broz et al. (2010)
Volatile emission	Enhanced/ shift in time	Pollination	Pollinators	Not shown for OD yet
Herbivore responses (directly to OD)				
Attraction to diverse odour bouquet	Enhanced	Locate diverse patches	Habitat, quality host plants, refuges	Hambäck et al. (2003), Held et al. (2003)
Repellence by diverse odour bouquet	Enhanced	Avoid diverse patches	Host plant	Khan et al. (2000), Mauchline et al. (2005), Nottingham et al. (1991)
Masking of host plant odours	Enhanced	Reduced host plant location	Host plant	Nottingham et al. (1991)
Search or discrimination activity	Enhanced	Compensation	Host plant	Wäschke et al. (2014), De Marco and Farina (2003)
Visual orientation	Enhanced	Compensation	Host plant	Not shown for OD yet
Herbivore responses (indirectly through the effect of OD on host plant)				
Repellence/ masking by adsorbed volatiles	Enhanced	Associational resistance, reduced abundance	Host plant	Himanen et al. (2010)
Preference of altered volatiles	Enhanced	Enhanced abundance	Host plant	Not shown for OD yet
Avoidance of altered volatiles	Enhanced	Reduced abundance	Host plant	Ninkovic et al. (2013)
Negative response to altered food quality of host plant	Enhanced	Associational resistance, reduced abundance	Host plant	Not shown for OD yet

(continued)

Table 6.1 (continued)

Responses to odour diversity (OD)	Direction of response to OD	Suggested ecological effect	Target of responder	Reference
Parasitoid and predator responses to OD				
Attraction	Enhanced	Location of diverse habitat patches	Habitat, nectar/food, quality hosts, refuges	Ninkovic and Pettersson (2003)
Masking, ignoring, avoidance, repellence	Enhanced	Reduced efficiency, abundance	Host plant/ host	Gohole et al. (2003), Randlkofer et al. (2007)
Preference of simple odour over diverse odour	Enhanced	Switch between habitat and close range host location	Host plant/ host	Wäschke et al. (2014)
Learning	Enhanced	Compensation	Host plant/ host	Vet et al. (1998)
Arthropod responses to pheromones and OD				
Masking, repellence	Enhanced	Reduced efficiency, avoidance of non-host plants	Sexual partner, conspecific	Byers et al. (2004)
Change of position	Enhanced	Increased detectability	Sexual partner, conspecific	Not shown for OD yet

plant compounds identified to date (Ober 2005), about 1700 volatile compounds have been detected by headspace analyses (Dudareva et al. 2006; Knudsen et al. 2006). The odour diversity is expected to be higher in diverse plant habitats since different plant species contribute different volatile patterns to the overall habitat odour (e.g. Courtois et al. 2009). However, due to the impact of different biotic and abiotic variables (see Sect. 6.2.2), the relationship between plant species diversity and odour diversity may be difficult to predict in the field (Randlkofer et al. 2010).

6.2.2 Abiotic Factors

Odour diversity in the field is highly dependent on abiotic factors that change plant volatile emission (e.g. Peñuelas and Llusià 2001). While a higher temperature is often known to increase plant volatile emission, Farré-Armengol et al. (2013) found that the response of floral emissions to temperature differed among species and among different compounds within the species. Warming not only increased the total rates of emissions but also changed the ratios of the compounds that constituted the floral scents. Nutrient deficiency usually reduces the emission of plant volatiles (Holopainen and Gershenzon 2010), leading to a reduced volatile diversity. However, plant diversity increases on nutrient-poor soils and might counterbalance the reduction of volatiles (Tilman 1988).

6.2.3 Biotic Factors

Besides pure species richness and abiotic influences on odour diversity, the range of different biotic interactions, e.g. between plants and plants, plants and arthropods and plants and microbes, also contributes to odour diversity (Ponzio et al. 2013). The plant species community in the vicinity can have profound effects on a nearby plant's metabolome (Scherling et al. 2010), which includes volatile production. Plant–plant competition (Agrawal et al. 2006; Barbosa et al. 2009), shading (Ballare 2014) or volatile-mediated induced responses in the focal plant (Ninkovic et al. 2013; Turlings and Ton 2006) are important influences on plant chemical composition and can act both below- or aboveground (Kos et al. 2015). The composition of the surrounding vegetation may not only influence the volatile emission of a single plant (e.g. Pierik et al. 2014), but the vegetation structure may also impact the habitat odour as it may affect the adsorption and rerelease of volatiles by plants (Wäschke et al. 2013). When attacked by herbivores, plant volatile emissions can vary depending on herbivore species, infestation time and herbivore density (Cai et al. 2014), and multiple attack can lead to nonadditive effects on the emitted volatile blend (Dicke et al. 2009; Pareja and Pinto-Zevallos, Chap. 3). And herbivores, endophytes, pathogens, mycorrhiza and bacteria can influence plant volatile emissions (Huang et al. 2003; Jallow et al. 2008; Piesik et al. 2011; Rapparini et al. 2008). Plant ontogenetic changes can also cause intrinsic variability in plant secondary metabolite emission by changes in blend proportions over time (Johnson et al. 2004). Plants in the vegetative and flowering stages produce different odours (Lucas-Barbosa et al. 2011), and plants emit more herbivore-induced volatiles while in their vegetative stage compared with individuals that already carry seeds (Rostas and Eggert 2008). Depending on the phenology of the plants in the habitat, the odour diversity of the vegetation might change drastically, e.g. arthropods orienting in flowering meadows might thus encounter high plant odour diversity. This has to be considered, for example, when studying herbivore–parasitoid interactions, where most experiments have been conducted with plants at the vegetative stage.

6.3 Effect of Odour Diversity on Plants and Arthropods

6.3.1 *Focal Plants*

While the effect of odour diversity on arthropods has been tackled by basic and applied research to some extent, its effect on plants has just started to be considered. Plants are able to sense their environment, and volatile cues may be one of several ways in which they gather information about neighbours and respond with appropriate morphological and physiological responses (Kegge and Pierik 2010; Ninkovic 2003). Diverse odours in the surroundings transfer different ecological information to a focal plant and might cause different responses. First, vegetation composition in terms of plant species diversity determines the abiotic and biotic environment of an individual plant and, thus, its growth, development and primary and secondary chemistry (Barbosa et al. 2009; Broz et al. 2010; Kigathi et al. 2013; Scherling et al. 2010). Plants neighbouring a focal plant can change the availability of nutrients in the habitat or alter its microclimate, influencing light intensity and temperature within a microhabitat. This may also change competition levels and the growth potential of the focal plant (Barbosa et al. 2009), which can obtain information on whether the neighbouring plants are a competitive threat and can accordingly adjust its growth (Ninkovic 2003). Second, a focal plant might be harder to detect by herbivores or pollinators when diverse surrounding odour masks its own bouquet. While in the case of lower predation risk, it might respond by reducing primary and secondary defences to allocate resources to other traits (e.g. growth and reproduction), in the case of pollinator attraction, it might enhance its volatile production or adjust it qualitatively to enhance its attractiveness to pollinators. The focal plant might also shift the time of volatile signal emission to periods where the surrounding plants are ‘quiet’.

The responses of the focal plant may also depend on its ‘lifestyle’, e.g. whether it is a pioneer plant settling in a diverse plant neighbourhood or whether it usually grows in more or less homogeneous plant and odour surroundings in older succession stages of the vegetation. Even conspecifics showing a differing genotype can cause a diverse odour environment for a focal plant and affect responses that cascade to higher trophic levels (Glinwood et al. 2009).

The neighbouring plant diversity can affect a focal plant above- and belowground (Kos et al. 2015). Plant individuals might be informed about the presence of heterospecifics either via airborne plant-emitted volatiles, compounds in the soil or via the mycorrhizal system (Heil and Karban 2010; Kegge and Pierik 2010; Selosse et al. 2006) and respond with the production of allelopathic compounds. Concentrations of the defensive compounds aucubin and catalpol in leaves of the plantain, *Plantago lanceolata*, correlated positively with plant species richness surrounding the plant in the field (Wäschke et al. 2015). Since plant iridoid glycosides can act allelopathically against heterospecific plant neighbours and inhibit seed germination, the presence of heterospecific competitors might lead to an increase in defensive compound concentrations and enhance the competitive ability of the focal plant against heterospecific neighbours. It needs to be shown in future studies whether the focal plant responds to volatiles or other neighbouring plant-mediated direct or indirect traits.

While the neighbouring plant diversity can affect a focal plant, the focal plant might also turn the tables and affect the neighbouring (odour) diversity, e.g. via inducing plant volatiles (Broz et al. 2010), adsorption (Himanen et al. 2010) or changing the soil environment (Iason et al. 2005) (Fig. 6.1).

6.3.2 Arthropods

All animals are confronted with a plethora of environmental stimuli during their search for food, sexual partners, places for reproduction or shelter. Volatile cues released from the habitat or from the food plant, prey or host itself play a key role in the foraging process of herbivores, predators and parasitoids. However, these resource-indicating odours encountered by arthropods in their natural environment need to be detected against a background that consists of a huge variety of other environmental odours and are often both diverse and variable mixtures themselves (Hilker and McNeil 2008). An important question is how arthropods cope with the problems of diversity and variability of the infochemical web.

6.3.2.1 Herbivores and Host Plant Signals

Direct effects of neighbouring plants on arthropods searching for a focal host plant can be mediated by volatiles that mask the focal plant or act as repellents or attractants (Mauchline et al. 2005; Nottingham et al. 1991; Randlkofer et al. 2007; Schröder and Hilker 2008; Tahvanainen and Root 1972; Wäschke et al. 2013). When foraging for host plants, herbivores have to cope with those non-host plants and diverse odour bouquets formed in diverse plant communities (Ponzio et al. 2013; Randlkofer et al. 2010). So far, the effects of diverse odour backgrounds on the foraging behaviour of arthropod herbivores appear to depend on the plant–herbivore system considered. Khan et al. (2000) showed that it is possible to exploit chemical ecology and plant species diversity for biological control of stem borers for maize and sorghum in Africa. Here planting molasses grass or silverleaf or greenleaf plants repelled the pest insects.

Hambäck et al. (2003) conducted field and laboratory experiments to elucidate the role of surrounding non-host plant vegetation on host plant finding by two chrysomelid and one curculionid beetle species. In olfactometer tests it was confirmed that the odours of the host plant, *Lythrum salicaria*, were only attractive to the curculionid beetle, *Nanophyes marmoratus*. Odours of the non-host plant, *Myrica gale*, were attractive for both tested chrysomelid beetle species, *Galerucella calmariensis* and *Galerucella pusilla*, but not for the curculionid beetle. However, in the field both chrysomelid beetles were less abundant and deposited fewer eggs on *L. salicaria* host plants that were surrounded either by *M. gale* non-host plant thickets or artificial plant models. In contrast, the curculionid species, *N. marmoratus*, was not distracted by neighbouring non-host vegetation or structures. The authors conclude that the non-host plant vegetation had visually masked the host plants from the two chrysomelid beetles, which seemed not to use plant odours for host location, whereas the curculionid was able to find the host plant within dense vegetation using olfactory host plant cues.

Wäschke et al. (2014) showed in laboratory bioassays that the herbivorous weevil *Mecinus pascuorum* can locate its host plant via olfactory cues even in the presence of non-host odour and enhanced odour diversity. The addition of non-host plant odours to host plant odour enhanced the weevils' foraging activity. In the field, plant diversity was positively correlated with weevil abundance indicating the weevil's sensory capacity to successfully cope with odour diversity when searching for hosts. In this case, odours emitted by diverse vegetation may represent a patch of interest and increase searching activity of foraging insects. Patches with host plants and high non-host plant diversity in the surroundings may provide enhanced host plant quality or offer refuges allowing herbivores to escape from natural enemies or competitors. Honeybee foragers respond to enhanced odour diversity by increased begging activity possibly to better learn relevant cues (De Marco and Farina 2003). For the whole herbivore community, an odour diverse habitat may have a stabilising effect as overpopulation of a single arthropod species could be prevented by olfactory disruption (Andow 1991; Wäschke et al. 2013). Salazar et al. (2016) found a negative correlation between chemical diversity in patches of *Piper* shrubs and plant damage by herbivory.

Indirect effects on the host plant may also be mediated by diverse vegetation stand odour leading to associational resistance, the protection of a focal plant from herbivory via neighbouring heterogenic species (Tahvanainen and Root 1972). As reported in Sect. 6.3.1, the volatile profile of a focal plant might be modified by adsorbing volatiles from neighbouring plants that can cause associational resistance when released again. This was shown for *Rhododendron* volatiles adsorbing to and repelling herbivores from neighbouring birch foliage (Himanen et al. 2010). A focal plant might also respond to volatiles from neighbouring plants by changing its blend (see Sect. 6.3.1), affecting specialised herbivores. Greater quantities of two terpenoids were found in the headspace of potato previously exposed to volatiles from undamaged onion plants. The altered potato volatile profile deterred winged *Myzus persicae* aphids in laboratory experiments, while companion planting of potato together with onion in the field reduced the abundance of winged, host-seeking aphids (Ninkovic et al. 2013). Indirect effects of odour from neighbouring plants, such as associational resistance mediated by volatile adsorption or induction or production of defence compounds, might be more common in diverse vegetation stands.

Since plants surrounded by conspecifics might suffer more from specialist herbivores than plants surrounded by heterospecifics, they might also invest more in defence against herbivores when growing in monoculture (Root 1973). Broz et al. 2010 found higher levels of total phenolics in methyl jasmonate-induced (herbivore-mimicked) *Centaurea maculosa* Lam. (Asteraceae) grown in monoculture than in plants grown with heterospecific neighbours. Therefore, the vegetation surrounding an individual plant can, besides other factors such as soil type, alter the plant's quality as a food source for herbivores and can influence higher trophic levels.

6.3.2.2 Odour Diversity and Predators and Parasitoids

The foraging efficiencies of many parasitoids and predators are reduced in more complex habitats, and stronger host–plant cues are required when habitat complexity increases (Bezemer et al. 2010). For example, eulophid egg parasitoids (Randlkofer et al. 2007) and tachinid flies (Gohole et al. 2003) are attracted by their respective host plant odours, but fail to respond or show a weakened response when the host plant odour is combined with deterrent non-host plant odours.

To cope with odour diversity during host plant and host location, parasitoids and predators employ different strategies such as ignoring, preferring or avoiding chemically complex environments (Hilker and McNeil 2008; Wäschke et al. 2013). They have developed different behavioural, sensory and neurophysiological adaptations to successfully locate their hosts in heterogeneous microhabitats, in habitats or even in the landscape as a whole. Parasitoids and predators can even benefit from orienting towards a more diverse odour indicating the host habitat, the presence of nectar as food or the refuges from their enemies (e.g. hyperparasitoids, intraguild predators).

Adult seven-spotted ladybirds, *Coccinella septempunctata*, respond to volatiles indicating plant diversity and identity of the habitat (Ninkovic and Pettersson 2003). The frequency of the predators was higher in barley plots containing high densities of the common weeds *Cirsium arvense* (L.) Scop. and *Elytrigia repens* (L.) Nevski than in control plots with only barley. In olfactometer experiments and in the absence of hosts and food, adult ladybirds showed a significantly more positive response to mixed odours of barley and each of the two weeds than to barley alone. Since ladybirds responded differently to barley plants that were previously exposed to volatiles from the two weeds, olfactory cues and plant–plant communication from diversified plant stands seem to be important mechanisms in predator attraction to sites with complex vegetation diversity. It is not only heterospecific plant diversity that positively affects adult ladybird orientation to odour from barley; within-species plant genotype mixing resulted in odour attraction/arrestment by *C. septempunctata*, and the ladybird was observed in greater numbers in mixed genotype stands in the field (Ninkovic et al. 2011).

Plant volatile diversity might differently affect generalist and specialist parasitoids and predators (Wäschke et al. 2013). Assuming that the variability in the learning process is adjusted to the variability in the chemical environment experienced by parasitoids and predators, specialists should utilise innate responses to chemical stimuli from the host plant, while generalists should employ learning processes during host foraging to cope with a high variability in host plant odours

(Vet et al. 1995). Thus, generalist parasitoids and predators might also use their learning ability during host (plant) search to cope with enhanced habitat odour diversity from many diverse plant species. The braconid wasp *Microplitis croceipes* hunts for a polyphagous larva and can learn to respond to individual compounds following experience with an odour mixture (Meiners et al. 2003). However, for certain compounds of a mixture, the olfactory background can affect recognition of individual compounds. This sensory filtering might explain why parasitoids cope with certain odorous environments better than with others during host searching. Vet et al. (1998) showed that the parasitoid *Leptopilina heterotoma* can adjust its degree of discrimination between similar odours of the substrate according to the profitability of the information in terms of the host encounter rate on the respective substrate. Predators and parasitoids might also adjust their learning effort concerning the discrimination between host plant and background odour diversity according to profitability of the information 'hidden' in the background odour.

Responses to enhanced odour diversity can also depend on the position of the parasitoid/predator in the landscape—whether it is searching for a suitable habitat or for host plants within a habitat. As was shown for different stem borer parasitoids and lacewings, the presence of non-host plants does not necessarily affect close-range foraging activities even though it has a long-range effect (Gohole et al. 2005; Salamanca et al. 2015). *Mesopolobus incultus*, a larval parasitoid of the weevil *Mecinus pascuorum* was attracted by a combination of host plant and host volatiles in both the absence and presence of non-host plant volatiles. In dual choice tests, the parasitoid preferred the blend of host plant and host volatiles over its combination with non-host plant volatiles and enhanced odour diversity; in the field, plant diversity did not affect parasitoid abundance (Wäschke et al. 2014). The parasitoid may use habitat odour for long-range orientation and may respond specifically to the pure host complex at a short-range scale when a choice between odours of the host complex and the surroundings is possible.

6.3.2.3 Odour Diversity and Pheromones

Does plant odour diversity differently affect the response of arthropods to plant signals or pheromones/host kairomones? There are some similarities in the spreading of both signal classes (Beyaert and Hilker 2014) but also differences. The simultaneous presence of pheromone and plant odours could either help to find a mate or conspecific, mask the female pheromone or be neutral, without any effect on the female- or conspecific-emitted pheromone (Deisig et al. 2014). There is evidence that the behavioural response of males to sex pheromone is increased by host plant volatiles (Reddy and Guerrero 2004). But what happens when a female or conspecific calling on a host plant is surrounded by increased odour diversity? Bark beetles' attraction to aggregation pheromones can be significantly reduced in the presence of volatiles from the leaves or bark of non-host trees (Byers et al. 2004). Large amounts of plant odours might represent a non-specific and highly variable odour landscape when a male insect is trying to orient towards small amounts of sex or aggregation pheromone. Deisig et al. (2014) discuss whether asynchronous emission of pheromones and plant odours might enable males to extract the quality, intensity and temporality of the pheromone signal from a diverse plant odour environment. Pheromone-releasing females might respond to the absence of males by changing their location, and it is known that many insects move to prominent places when searching for partners (Alcock 1987).

6.4 Conclusions

The ecological relevance of the outcome of interactions between host plant (or pheromone) and diverse vegetation stand odour information is still under discussion and most likely depends on the plant/arthropod species, the environmental context and the precise compounds involved. Arthropods might be able to cope with vegetation stand odour diversity when the host plant species' odour blend differs sufficiently from the surrounding vegetation stand odour or when the vegetation structure interacts with abiotic conditions (e.g. wind) in a way that the host plant blend or pheromones can be perceived as distinct odour. In Sect. 6.3.2.2, the connection between parasitoid and predator generalist lifestyle, the learning ability and the capability to cope with vegetation stand odour diversity has been outlined. Also the reproductive strategy of an arthropod might predestine its ability to respond to vegetation stand odour diversity. For example, koinobiont parasitoids living on feeding and growing parasitised hosts will benefit from assessing host plant chemistry by olfactory cues as a predictor of host performance and by discerning it from vegetation stand odour diversity. Another trait of arthropods influencing their movement or learning abilities in complex odour surroundings is body size. Very small arthropods (e.g. some egg parasitoids) are restricted to passive habitat searching due to their low dispersal and flight abilities and might ignore vegetation stand odour diversity. Moreover, brain size might determine the learning ability of vegetation stand odours (Fatouros et al. 2008).

Studying the ecological role of odour diversity has just begun, and researchers trying to unravel the orientation mechanisms of arthropods in diverse 'odour landscapes' will need to develop novel tools to succeed. When testing arthropods from different trophic levels for their response to odour diversity, different olfactometers might be needed, since the size of an arthropod, its morphology, flight behaviour and its neurophysiology determine its olfactory-mediated foraging behaviour. Research on arthropod responses to diversity of plant volatiles is dominated by small-scale olfactometer experiments in the lab. We need new bioassays in the lab simulating the field situation. Wäschke et al. (2014) developed a circle arena where the focal plant is under the inner circle, while the neighbouring plants can be placed in the outer circle and the arthropod can experience odours from the host plant through a cloud of non-host plant odours. For situations involving mild airflow, it would require the construction of a dynamic olfactometer taking into account the structure of the plants. In the field the influence of odour diversity on the organisms may be measured by observational and experimental studies. Focal and adjacent plants as well as dispensers with defined mixtures of compounds can be arranged in a certain way to reveal the impact of odour diversity.

Wilson et al. (2015) describe how odorous noise shapes the efficacy and evolution of biological communication systems and how noise affects each piece of the signalling chain (generation, transmission and reception) in olfactory communication. They outline how information theory provides methods for analysing chemical communication and hypotheses for how selection might act to reduce noise. At a later stage, we might use the novel knowledge gained by new experimental and theoretical approaches when designing ‘odour landscapes’ favourable for nature conservation or biological control of pest species.

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Chapter 7
Neighbour Recognition Through Volatile-Mediated Interactions

Tao Li

Abstract Plants constitutively emit a wide array of volatile organic compounds (VOCs) and, upon biotic and abiotic stress, release a more complex and more diverse VOC blend. These VOCs mediate multiple ecological interactions between plants and their associated community members, including plant–plant communication or neighbour recognition. Albeit initially discredited, the concept of VOCs mediating plant–plant communication is now well accepted. In general, plants perceive and respond to VOCs emanating from their neighbours with physiological, biochemical or phenotypic changes that may convey resistance to abiotic and biotic stress. However, the mechanisms underpinning this process, the ecological and evolutionary relevance as well as the circumstances under which this process occurs remain largely obscure. In particular, there is very scarce information on whether and how global change, which has increasingly been shown to change VOC emission patterns and alter VOC atmospheric lifetimes, can disrupt VOC-mediated plant–plant communication. This chapter updates our current knowledge about these aspects and, through synthesising them, intends to point out gaps in existing research, in particular the need for further studies in a changing environment.

7.1 Introduction

Unlike animals, plants are rooted, sessile organisms. Although they can grow towards the sun and bend with gravity, they cannot migrate in search of food or a mate nor escape danger from their predators. As such, plants have to develop incredibly sophisticated and complex sensory mechanisms that would allow them to survive in their ever-changing environments. Indeed, scientists have long

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recognised that plants can sense and respond to changes in their environments, integrating and evaluating diverse information and then adjusting their physiology, morphology and phenotype accordingly (Karban 2008; Pierik and De Wit 2014; Kessler 2015). For example, they recognise bad neighbours and compete with them for above- and belowground resources such as light, water and nutrients (Pierik and De Wit 2014). They communicate with a myriad of other organisms surrounding them, such as insects and animals that pollinate them and help to disperse their seeds, predatory and parasitic insects that kill the attacking herbivorous insects, and microbes that facilitate the nutrient acquisition or confer resistance to disease and herbivores (Heil 2014; Kessler 2015). These capabilities allow plants to expand and protect their territory. In addition, plants can perceive themselves and communicate with one another, warning other parts of the same plants or neighbouring plants of the same or different species that there is trouble on the way (Farmer 2001; Karban 2008; Karban et al. 2010). This form of plant communication often occurs either through volatile chemicals transmitted in the air (Karban et al. 2010) or via soluble compounds exchanged by roots and mycelial networks in the rhizosphere (Johnson and Gilbert 2015), although alternative routes, for example, through sound (Gagliano and Renton 2013), are also possible in some cases. This chapter will be restricted to volatile-mediated plant–plant communication.

Our awareness of plant communication through volatile cues dates back to 1983 when two research groups (Baldwin and Schultz 1983; Rhoades 1983) working independently in two different laboratories discovered almost simultaneously that plants grown close to damaged neighbours became more resistant or chemically more defended against herbivores than those grown farther away or nearby undamaged neighbours. Since then, this phenomenon has been widely called ‘talking trees’, plant–plant communication or plant–plant signalling to describe that herbivore damage causes plants to emit volatile cues that neighbouring plants respond to (Heil and Karban 2009; Karban et al. 2014b). Albeit initially criticised severely, the concept of plant–plant communication via volatiles is now widely accepted and has been applied in agriculture to tweak agricultural practices and make crops better defended against pests. Because the field of plant–plant volatile communication has been extensively reviewed recently (Das et al. 2013; Blande et al. 2014; Pierik et al. 2014; Karban et al. 2014b; Yoneya and Takabayashi 2014), this chapter aims to provide an update on recent developments and advances in this field. The chapter begins with an overview of two basic types of volatile communication, namely, communication via stress-induced or constitutively emitted volatiles. This is followed by a review of the chemical identity of volatile signals that convey information between plants. This chapter then proceeds with a more detailed review of the circumstances where plant communication may occur or fail, especially under local and global change, and ends with suggestions for future research.

7.2 Plant Communication via Volatiles

7.2.1 Communication via Induced Volatiles

Plants release a wide diversity of volatile organic compounds (VOCs) into their surroundings, with the emission patterns changing remarkably with biotic and abiotic stresses (Dudareva et al. 2013; Kessler 2015; Ul Hassan et al. 2015). With regard to biotic stresses, herbivore attack or pathogen invasion usually induces plants to increase VOC emissions and/or de novo synthesise new compounds, although stress-induced suppression of VOC emissions could also occur in some cases. So far, more than 1700 volatile compounds have been identified as emitted by plants under various conditions, mainly consisting of green leaf volatiles (GLVs), terpenoids and benzenoids (Dudareva et al. 2013). These VOCs play an important role in the interactions of plants with their associated community members, including plants themselves and organisms at higher trophic levels (Heil 2014; Kessler 2015). For instance, herbivore-induced plant VOCs can attract natural enemies of the attacking herbivores—a phenomenon called ‘plants crying for help’ or indirect defence (Heil 2014).

Plants may not just emit VOCs to appeal for insect help when damaged—they may also make use of the same cues to warn each other of impending herbivore attack. Neighbouring plants can interpret chemical messages encoded in those volatile cues and ramp up their defence mechanisms in response. As mentioned above, plant–plant communication via herbivore-induced VOCs gained a high profile in the early 1980s, with the ‘talking trees’ theory popularised in both the scientific circles and the media. But the idea was quickly attacked, attracting criticism that the study was statistically flawed and irrelevant to the real-world struggle between plants and herbivores (Farmer 2001; Heil and Karban 2009). Since 1990, however, rigorous laboratory and field studies that overcame those early criticisms began to yield results in favour of VOC-mediated plant–plant communication (Farmer and Ryan 1990; Arimura et al. 2000a; Karban et al. 2000). So far, plant–plant volatile communication upon herbivore attack has been observed in more than 30 plant species that include herbaceous and woody plant taxa (Heil and Karban 2009; Karban et al. 2014b). According to a recent meta-analysis (Karban et al. 2014b), 40 out of 48 studies of plant–plant communication via herbivore-induced VOCs have found evidence of communication that affects herbivory, with laboratory or greenhouse studies dominating the literature and showing stronger induced resistance than field studies.

While volatile communication following herbivore attack has been more widely accepted over the past few years, much less is known about communication upon pathogen infection. The first evidence was published in 1997 when Shulaev et al. (1997) found that tobacco plants (*Nicotiana tabacum*) inoculated with tobacco mosaic virus resulted in adjacent healthy plants exhibiting greater induction of pathogen-related proteins and a lower percent of leaf necrosis than plants receiving air from mock-inoculated plants. After experiencing almost two decades of slow progress following the initial report, research on plant volatile communication upon pathogen assault has grown rapidly in the last decade. Thus far, convincing evidence of pathogen-induced VOCs mediating disease resistance in neighbouring plants has been found in many plant species, including *Arabidopsis* (Kishimoto et al. 2005), lima bean (Yi et al. 2009), maize (Piesik et al. 2011), wheat (Piesik et al. 2013; Ameye et al. 2015; Castelyn et al. 2015), barley (Piesik et al. 2013), oat (Piesik et al. 2013) and common bean (Quintana-Rodriguez et al. 2015), as well as in several types of pathogens such as pathogenic viruses (Yao et al. 2012), bacteria (Dorokhov et al. 2012) and fungi (Quintana-Rodriguez et al. 2015). However, it is worth noting that almost all of these studies were conducted in the laboratory, except the study with lima bean in which disease resistance induction by airborne cues was seen in the laboratory as well as in the wild. Therefore, how frequently this phenomenon may occur in nature remains contentious.

In addition to plant VOCs induced by biotic stress, VOCs induced by abiotic stress—which indicate the physiological status of stressed plants—might also trigger defence responses in recipient plants. This, however, has been appreciated only very recently in a few studies on plant responses to UV-C irradiation and salinity. In the case of UV-C irradiation, irradiated *Arabidopsis thaliana* or tobacco plants were shown to generate a volatile signal that led to genomic instability in neighbouring nonirradiated plants, a response that may be part of plant adaptation to UV-C irradiation (Yao et al. 2011). With regard to salt stress, *Arabidopsis thaliana* plants that experienced high salinity increased VOC emission, which in turn elicited high salt resistance in neighbouring plants (Lee and Seo 2014).

All in all there is compelling evidence showing that plants can communicate with each other about the current and future environmental conditions through stress-induced VOCs. Furthermore, as a response to volatile cues emitted by stressed neighbours, unstressed plants in close proximity may also emit a similar bouquet of VOCs that could potentially induce stress responses in additional unstressed plants located further away from the stressed plants. Such a chain of communication has been demonstrated in some systems (Muroi et al. 2011; Arimura et al. 2012) and suggests that the message transmitted by the original emitter plants would be able to reach neighbouring plants over a much larger area. So far, plant–plant communication via induced VOCs has been thought to work most often at distances of up to 1 m (Heil and Karban 2009; Karban et al. 2014b).

7.2.2 Communication via Constitutive Volatiles

In plant communities, plant VOCs are important infochemicals mediating plant–plant and plant–insect interactions. In principle, VOCs constitutively released from one healthy plant may affect interactions of a neighbouring plant with its herbivores through either directly repelling herbivores or indirectly through inducing physiological or biochemical changes in the neighbouring plant which in turn affect herbivore acceptance and performance. The first type of interaction is one example of VOC-mediated associational resistance and has been demonstrated in several plant species (Karban 2007; Underwood et al. 2014; Quintana-Rodriguez et al. 2015). The second type of interaction involves active responses of recipient plants and falls into the category of plant–plant chemical communication. Early evidence of chemical communication through constitutive VOCs was the recognition that under both laboratory and field conditions, VOCs from healthy plants of one barley (*Hordeum vulgare*) cultivar resulted in neighbouring plants of another cultivar becoming less acceptable to the bird cherry-oat aphid *Rhopalosiphum padi* (Pettersson et al. 1999; Ninkovic et al. 2002). Following this, similar observations were found in many other barley cultivars or genotypes as well as in the responses of barley to VOCs emitted from several weed species (Glinwood et al. 2004, 2011; Ninkovic et al. 2009). Moreover, these studies also showed that exposed barley cultivars not only became less acceptable to cereal aphids but also more attractive to aphid predators. Another example is the chemical interaction between onion and potato (Ninkovic et al. 2013). In this case, potato plants after exposure to VOCs from undamaged onion plants had an altered VOC profile, leading to increased deterrence of the aphid *Myzus persicae* and enhanced attraction of the ladybird *Coccinella septempunctata* (Ninkovic et al. 2013; Vucetic et al. 2014; Dahlin et al. 2015). These studies are important steps forward in understanding the mechanisms underlying chemical communication between undamaged plants.

However, the ecological reasons behind this process remain unclear. It seems counterintuitive that receiver plants perceive and respond to VOCs from unattacked neighbouring plants with anti-herbivore defence induction since VOCs constitutively released by healthy plants do not indicate any pressure of herbivory. It has been speculated that VOCs released by healthy emitters carry information on the emitters themselves and that receiver plants can acquire valuable information from these VOCs on whether their neighbouring plants are potential competitors and adjust their growth accordingly (Ninkovic et al. 2013). Evidence supporting this idea came from an early study on communication between two barley cultivars (Alva and Kara) showing that Kara altered the carbon allocation pattern as a response to VOCs from Alva, investing more biomass to roots (Ninkovic 2003). In addition, studies on ethylene-induced shade-avoidance response seem to lend further credence to this idea (Pierik and De Wit 2014). Ethylene, the first identified gaseous phytohormone, has been thought to serve as a volatile signal indicative of the presence of neighbouring plants competing for light (Pierik and De Wit 2014; Pierik et al. 2014). In dense stands, ethylene has been found to accumulate to an extent that induced stem elongation (De Wit et al. 2012; Kegge et al. 2013). Furthermore, some parasitic plants may exploit plant VOCs to locate host plants (Runyon et al. 2006; Kaiser et al. 2015). For example, Runyon et al. (2006) demonstrated that the parasitic plant dodder (*Cuscuta pentagona*) could discriminate between VOCs emitted by host and nonhost plant species and direct their growth towards the odours of host tomato plant (*Solanum lycopersicum*).

7.2.3 Priming and Induction

Exposure to biotic and abiotic stresses usually directly induces defence responses in exposed plants. However, plants may not activate a detectable defence under certain circumstances, for example, after a transient exposure to mild stress (Shiojiri et al. 2012). In this context, plants may recognise and remember an initial contact with the stress factors within a given period of time and respond more quickly and/or strongly the second time that they encounter them. Such responses have been referred to as 'priming and memory' in planta and have been demonstrated to be common in plant defence against pathogens and herbivores (Pastor et al. 2013; Balmer et al. 2015). Priming has also been reported in several plant species as a response to volatile cues emanating from stressed neighbours (Pastor et al. 2013). For example, maize plants previously exposed to herbivore-induced VOCs responded more strongly against subsequent herbivore attack by increasing jasmonic acid biosynthesis and VOC release than did unexposed plants (Engelberth et al. 2004). This was the first report on priming against insect herbivory signalled by plant VOCs. Since this initial discovery, the priming effect has been confirmed in many other studies (Heil and Kost 2006; Heil and Silva Bueno 2007; Ton et al. 2007; Frost et al. 2008; Li et al. 2012; Ali et al. 2013) and in more natural environments (Kessler et al. 2006; Heil and Silva Bueno 2007) and has extended to plant VOCs induced by pathogen infection and abiotic stress (Yi et al. 2009; Lee and Seo 2014; Ameye et al. 2015). Unlike induced defence, which is often associated with high fitness costs, priming can be triggered by relatively low amounts of VOCs from stressed emitter plants and does not exert high fitness costs for defence responses to receiver plants in the absence of stress (Pastor et al. 2013). Recent studies at different levels from transcriptomics to metabolomics to proteomics, together with bioinformatics tools, have started to shed light into the molecular aspects of priming (for details see a recent review by Balmer et al. 2015).

7.3 Chemical Identity of Signals in Plant Volatile Communication

The relatively low cost and widespread availability of gas chromatography–mass spectrometry (GC-MS) as well as commercial authentic standards have facilitated the identification of individual compounds in the often-complex VOC blends that dictate the content of the messages in plant–plant communication. Volatile compounds identified so far in this context consist of compounds belonging to different chemical classes (Fig. 7.1). They are fatty acid-derived oxylipins such as green leaf volatiles (*E*)-2-hexenal, (*Z*)-3-hexenal, (*Z*)-3-hexenyl acetate and (*Z*)-3-hexenol (Engelberth et al. 2004; Ruther and Kleier 2005; Kost and Heil 2006; Frost et al. 2008); octadecanoid compounds methyl jasmonate and (*Z*)-jasmone (Bruce et al. 2008; Oluwafemi et al. 2013); several terpenoids such as (3*E*)-4,8-diemthyl-1,3,7-

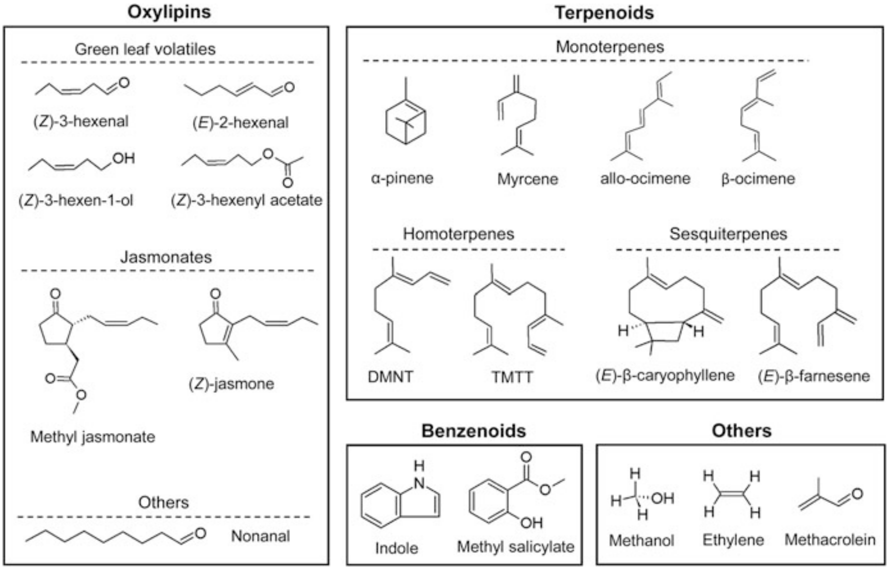

Fig. 7.1 Chemical structures of selected plant VOCs known to mediate plant–plant communication. DMNT: (3*E*)-4,8-diemthyl-1,3,7-nonatriene, TMTT: (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

nonatriene (DMNT), (3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) and β-ocimene (Arimura et al. 2000a,b; Godard et al. 2008); and two benzenoid compounds methyl salicylate (MeSA) and indole (Erb et al. 2015). A few other compounds such as methanol (Dorokhov et al. 2012), methacrolein (Kessler et al. 2006) and ethylene (Ruther and Kleier 2005) have also been shown to trigger a defence response in receiver plants against herbivores or pathogens. Among these, GLVs, which are almost ubiquitously produced by green plants, seem to be the best characterised and most conserved signals, exhibiting widespread activity in eliciting defence responses in various plant species at the genetic, metabolic or phenotypic levels (Scala et al. 2013; Ameye et al. 2015; Yamauchi et al. 2015). Since many empirical studies and reviews have been published on the biosynthesis and function of plant VOCs (Dudareva et al. 2013; Ul Hassan et al. 2015), in the following paragraphs, I will give a brief description of the biosynthetic pathways leading to the aforementioned compounds and the bioactivity in inducing plant defence responses.

GLVs, which characterise the ‘freshly mowed lawn’ smell, consist of a blend of saturated and unsaturated six-carbon aldehydes, alcohols and esters and originate through the oxylipin pathway from the C_{18} polyunsaturated fatty acids linoleic acid and α-linolenic acid, which are liberated from membrane lipids (Engelberth et al. 2013; Scala et al. 2013; Ul Hassan et al. 2015). These fatty acids are deoxygenated by lipoxygenase (LOX) to form hydroperoxides that act as substrates for several downstream competing pathways, including the hydroperoxide lyase

(HPL) and the allene oxide synthase (AOS) pathways. The former leads to the formation of GLVs, while the latter synthesises jasmonates. The synthesis of GLVs begins with the cleavage of 13-hydroperoxy octadecatrienoic acid (13-HPOTE) by HPL to form (*Z*)-3-hexenal, which is enzymatically converted to other C_6 compounds, including (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate. Healthy, undamaged plants usually emit trace amounts of GLVs, but increase the emission rapidly and substantially upon herbivore or pathogen assault and may sustain the increased emission over the period of, and even after, herbivore feeding or pathogen infection (Engelberth et al. 2013; Scala et al. 2013). Such properties indicate that, in addition to protecting injured plants from further damage, GLVs could serve as potential carriers of distress information. In fact, many studies using synthetic GLVs or transgenic plants that are deficient in synthesising GLVs have confirmed that GLVs have this function, inducing and/or priming defence mechanisms in distal parts of the same plants or neighbouring undamaged plants (Engelberth et al. 2004, 2013; Kost and Heil 2006; Frost et al. 2008). For example, (*Z*)-3-hexenyl acetate elicited plasma membrane potential depolarization and cytosolic calcium (Ca^{2+}) flux in tomato and *Arabidopsis* (Asai et al. 2009; Zebelo et al. 2012), expression of defence-related genes (Engelberth et al. 2013), accumulation of defensive chemicals (Frost et al. 2008), delay of disease development (Ameye et al. 2015) as well as reduced herbivore damage and increased production of inflorescence (Kost and Heil 2006; Heil and Silva Bueno 2007). Nonanal, which is presumed to be derived from the non-enzymatic oxidation of oleic acid, has been shown to induce or prime plant resistance against pathogens (Girón-Calva et al. 2012).

Similar to GLVs, the formation of methyl jasmonate (MeJA) begins with LOX-derived 13-HPOTE, which is catalysed into allene oxide by AOS and subsequently transformed into a cyclopentenone by allene oxide cyclase (Farmer and Ryan 1990; Tamogami et al. 2008). Apart from playing important roles in diverse developmental processes such as seed germination, root growth, flowering and senescence, MeJA has also been described repeatedly to induce and prime plants to activate a large number of defence genes and defence-related proteins (e.g. proteinase inhibitors) as well as toxic or repelling secondary metabolites (e.g. nicotine, VOCs) (Farmer and Ryan 1990; Tamogami et al. 2008; Kegge et al. 2013). (*Z*)-jasmone is postulated to originate from the octadecanoid pathways. Similarly, exposing plants to physiological concentrations of (*Z*)-jasmone induced and/or primed VOC emission, which in turn rendered exposed plants more repellent to herbivores and more attractive to predators and parasitoids (Bruce et al. 2008; Delaney et al. 2013; Oluwafemi et al. 2013).

Plant terpenoids are the largest and most diverse class of secondary metabolites with many volatile constituents, derived from two common five-carbon precursors isomethylallyl pyrophosphate and its isomer dimethylallyl pyrophosphate. In plants, both the plastidic methylerythritol phosphate (MEP) and the cytosolic mevalonic acid (MVA) pathways are responsible for the formation of these C_5-isoprene building units. The MEP pathway usually generates volatile hemiterpenes (C_5) and monoterpenes (C_{10}), while the MVA pathway often produces volatile sesquiterpenes (C_{15}) (Dudareva et al. 2013). The biosynthesis and emission of volatile terpenoids are not only developmentally and diurnally regulated but also affected by many abiotic and biotic factors (Dudareva et al. 2013). Plant terpenoids play important roles in protecting plants from abiotic stress as well as in mediating multitrophic interactions between plants and insects at other trophic levels, including plant–plant communication (Godard et al. 2008; Dudareva et al. 2013). Although so far several volatile terpenoids have been suggested to be implicated in plant–plant communication, research on the potential physio-biological activities of individual terpenoid compounds has not received the same attention as GLVs. (*E*)-β-ocimene, allo-ocimene, DMNT and TMTT induce expression of several defence genes in lima bean (Arimura et al. 2000a, b, 2012). Among these terpenoids, (*E*)-β-ocimene is the best-studied compound triggering defence responses in exposed plants and seems to be a conserved signal triggering defence responses (Arimura et al. 2012; Cascone et al. 2015). Exposing either lima bean or maize in an open-flow tunnel to (*E*)-β-ocimene emitted by transgenic tobacco constitutively overexpressing (*E*)-β-ocimene synthase primed VOC emission in exposed plants (Arimura et al. 2012). As such, primed lima bean plants received a reduced egg load by spider mites and became more attractive to predatory mites; primed maize had reduced larval growth (*Mythimna separata*) and enhanced attraction of parasitic wasps. In line with these findings, a very recent study with tomato showed transgenic plant that produced (*E*)-β-ocimene directly induced VOC emission and consequently reduced aphid (*Macrosiphum euphorbiae*) acceptance and development while augmenting aphid parasitoid (*Aphidius ervi*) attraction (Cascone et al. 2015).

With the exception of MeSA, benzenoid compounds have received little attention as potential volatile signals. Only MeSA and indole have been identified to engage in plant–plant communication (Shulaev et al. 1997; Erb et al. 2015). Both compounds are derived from the shikimate pathway (Dudareva et al. 2013). MeSA has long been known to elicit systemic acquired disease resistance. Several studies showed that external exposure to MeSA either induced or primed disease resistance (Shulaev et al. 1997; Girón-Calva et al. 2012). Indole is a volatile compound that has been recently shown to convey information within and between plants. It is synthesised from indole-3-glycerol phosphate by the indole-3-glycerol phosphate lyase and is emitted from many plant species, especially in response to herbivore attack (Erb et al. 2015). Using synthetic indole or indole-deficient mutants, a recent study showed that indole primed VOC emissions as well as jasmonic acid and abscisic acid production in both systemic leaves and neighbouring maize plants (Erb et al. 2015).

Ethylene has been shown to reduce shade-avoidance responses (Pierik and De Wit 2014; Pierik et al. 2014). Studies found that herbivore attack induced the ethylene burst in native tobacco, which in turn reduced nicotine production but did not affect herbivore-induced VOC emission (von Dahl et al. 2007). In maize, exogenous application of ethylene revealed that it can synergise VOC emissions induced by (*Z*)-3-hexenol (Ruther and Kleier 2005). Methanol originates from the demethylation of pectin within the cell wall by pectin methylesterases, and its emission can be induced by herbivore attack or pathogen infection (von Dahl et al. 2006). Exposing plants to methanol has been shown to increase plant resistance to pathogens (Dorokhov et al. 2012). Methacrolein, one of two major products resulting from the reaction of isoprene with OH radical in the atmosphere, has been shown to occur in the essential oil of certain plants, for example, sagebrush, and exposing native tobacco plants to this compound primed production of trypsin proteinase inhibitors (Kessler et al. 2006).

While the aforementioned compounds individually induce or prime defence responses, such induction or priming is species specific. Some compounds show stronger inductive capability than others (Godard et al. 2008; Zebelo et al. 2012). Whether individual compounds or the full blend are essential for inducing defence responses in nature remains obscure and awaits future research. However, some studies have shown that the whole volatile blend does matter in inducing responses (Kikuta et al. 2011; Ueda et al. 2012). For example, (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate and (*E*)-β-farnesene, five key components of wound-induced volatile blends in *Pyrethrum* daisy (*Tanacetum cinerariifolium*), were effective in inducing biosynthesis of insecticidal pyrethrins only when applied in a mixture but not individually (Kikuta et al. 2011).

7.4 The Context Dependence of Volatile-Mediated Plant–Plant Communication

Plant–plant communication via volatiles has convincingly been shown to affect the resistance profiles of receiver plants. However, the effectiveness of plant–plant communication hinges on many factors, including plant species identity (Karban et al. 2004; Karban 2007; Pearse and Karban 2013; Pearse et al. 2012, 2013), plant genetic relatedness (Karban and Shiojiri 2009; Karban et al. 2013), plant age (Shiojiri and Karban 2006), timing and intensity of herbivore attack (Shiojiri and Karban 2008a; Shiojiri et al. 2009), duration of the signal transmission (Shiojiri et al. 2012), herbivore identity and traits (Hughes et al. 2015) and air quality (Blande et al. 2010; Li and Blande 2015). It is well known that different plant species release a distinct blend of volatile compounds and that both the quantity and quality of volatile compounds change depending on abiotic and biotic factors. Such differences dictate the privacy of the volatile language. The emitter plants are expected to broadcast distress signals to associated community members that help themselves or their relatives, and receiver plants should sense and respond to the distress signals that are reliably indicative of the potential risk of impending danger. In doing so, the emitter plants would minimise other irrelevant organisms eavesdropping on the distress signal, while the receiver plants would avoid responding to dishonest distress signals. In this context, one could imagine that natural or artificial selection pressure may cause plants to develop species-specific or context-specific between-plant volatile signals, a process that could lead to signal diversification in nature.

7.4.1 Plant Species Specificity in Plant–Plant Communication

Plant–plant communication can occur both within and between species, though the latter occurs only in certain species combinations. However, both intraspecific and interspecific communication seems to be plant species specific as some plants respond to volatile cues released from their damaged neighbours, but others do not. A remarkable example concerns chemical communication between sagebrush and its neighbouring plants of the same or other species. Wild sagebrush plants downwind from conspecific neighbours damaged either by artificial clipping or herbivore feeding consistently experience less leaf loss to herbivores than those with undamaged neighbours (Karban et al. 2004, 2006). A similar response manifests itself in both wild and experimentally transplanted tobacco plants when grown in close proximity of damaged sagebrush in the field (Karban 2001; Karban et al. 2000, 2003). In the laboratory, potted tomato plants that are sealed in an airtight glass vessel together with clipped sagebrush foliage become chemically more defended as indicated by the increased production of proteinase inhibitors (Farmer and Ryan 1990), chemicals that can adversely affect herbivore performance. However, four other wild plant species that live close to, and share the same generalist herbivores with sagebrush, do not alter resistance profiles when neighbouring sagebrush plants are experimentally clipped (Karban et al. 2004; Karban 2007). Even in the genus *Artemisia*, no evidence of plant communication is found between silver sagebrushes (*Artemisia cana*), though such communication occurs in California mugwort (*Artemisia douglasiana*) as in sagebrush (Shiojiri and Karban 2008b). The effectiveness of volatile-mediated plant–plant communication may be far more species specific within a multispecies neighbourhood, where the response is not only affected by the relatedness of the neighbours but also by the herbivore species encountered. In a large, manipulative field study using three annual herbaceous plant species (*Achyrachaena mollis*, *Lupinus nanus* and *Sinapis arvensis*), evidence that damaging neighbours results in induced resistance in receiver plants was only found in the Brassicaceae species *S. arvensis* in a laboratory feeding trial in which receiver plants received less leaf damage by a specialist herbivore *Pieris rapae* (Pearse et al. 2012). Yet, in a field survey, leaf damage of receiver plants by naturally occurring herbivores was not affected by whether neighbouring plants were damaged or not. For the other two species, evidence of plant communication was not found in either the laboratory or field.

Not only do plant responses to herbivore-induced volatiles display plant species specificity, volatile communication between undamaged plants is also species-combination specific. For example, work on volatile chemical interaction between barley and weeds showed that barley plants exposed to VOCs from the weed *Chenopodium album* became less acceptable to the bird cherry-oat aphid (*Rhopalosiphum padi*) in both the laboratory and the field (Ninkovic et al. 2009). However, the effects of weed VOCs on barley–aphid interactions are limited to certain species and do not appear to be an ubiquitous phenomenon since only two of the 19 weed species tested were found to exert such effects in a laboratory screening test (Ninkovic et al. 2009). Furthermore, chemical exchange between undamaged plants is cultivar or genotype specific. Several studies on the effects of volatile chemical interaction between different barley cultivars or genotypes on aphids and their natural enemies have shown that volatiles from certain barley genotypes render certain other barley genotypes less acceptable to cereal aphids and more attractive to aphid natural enemies (Glinwood et al. 2009, 2011; Ninkovic et al. 2002; Ninkovic and Åhman 2009; Pettersson et al. 1999). These studies have also found that some genotypes, such as the older ones that were released to the market a long time ago, are more likely to act as responders, whereas other more recently released genotypes act most often as inducers.

7.4.2 Kin Recognition

Apart from plant species identity, genetic distance or relatedness between individuals within a plant species can impact the effectiveness of plant–plant communication. This has been clearly demonstrated in sagebrush, where plants that received volatile cues emitted from genetically identical clones experienced less natural damage over the growing season than plants that received cues from genetically distant clones (Karban and Shiojiri 2009; Karban et al. 2013). Likewise, a field study on willow, the first system that disclosed controversial evidence of VOC-mediated plant–plant communication, also showed that the perception of volatile cues from close relatives reduced foliar damage more effectively compared with cues from distantly related individuals (Pearse et al. 2013). These studies collectively suggest that plants can respond differently to cues from kin, reacting more effectively to self than non-self volatile cues. Kin recognition in plants was first found to occur through root contact or root exudates. In this case, plants grew roots or stems less aggressively when their roots encountered relatives compared with strangers (Karban et al. 2013). Studies with sagebrush and willow have revealed that kin recognition can also take place through airborne volatile cues. However, it is still too early to generalise how common this phenomenon is before more plant species are studied. Interestingly, a recent study showed that plants may even utilise volatile chemotypes to distinguish relatives from strangers. In this case, sagebrush plants of a similar volatile chemotype communicated more effectively with each other and accumulated less natural herbivore damage than plants of different chemotypes (Karban et al. 2014a).

7.4.3 Dose and Exposure Duration

While there remains little information on the precise concentrations of VOCs required for a receiver plant to trigger a defence response and the concentrations of emitter-derived VOCs that are reached in the headspace of the receiver, it seems intuitive that VOC-elicited responses in a receiver are related to the amount of VOCs (dose) that the receiver is exposed to as well as the exposure duration. Plants exposed over longer periods, or repeatedly to low concentrations of VOCs, may initiate a measurable response that may not be detectable after shorter or single exposure events (Girón-Calva et al. 2012; Shiojiri et al. 2009, 2012). For instance, exposure of lima bean to a low concentration of MeSA over 24 h led to significantly enhanced resistance to the bacterial pathogen *Pseudomonas syringae*, whereas exposure to the same concentration for 6 h failed to do so (Girón-Calva et al. 2012). In *Arabidopsis*, a clear molecular and physiological response was observed when plants were intermittently exposed over a period of 3 weeks to trace amounts (below 140 ppt) of GLVs emitted by freshly injured *Arabidopsis* plants, but not after only two exposure events in a single week (Shiojiri et al. 2012).

7.4.4 Plant Age and Seasonal Variability

It is known that plant defences can vary dramatically over time and space, presumed to be driven by plant ontogeny or by temporal and spatial changes in potential herbivory (Barton and Koricheva 2010; Desurmont et al. 2014). Since plant responses to environmental cues are adaptive only when these cues can truly forecast the environmental threats facing the receiver plant, chemical communication between plants is likely to be more effective at inducing defence responses under certain conditions. In sagebrush, experimentally clipping early in the season when plants were undergoing active growth and herbivory pressure was high was found to be more effective at causing neighbours to become more resistant than was later clipping (Shiojiri and Karban 2008a). Furthermore, young sagebrush plants were shown to be more effective at emitting and responding to volatile cues (Shiojiri and Karban 2006). Similarly, young, developing aspen leaves were also reported to be more responsive to volatile cues than mature leaves (Li et al. 2012).

7.4.5 Neighbour Recognition in a Changing Environment

VOC emissions are strongly dependent on environmental conditions. A major concern is whether or not the predicted global climate change will influence and modify plant–plant communication mediated by VOCs. Elevated atmospheric CO_2 and ground-level O_3, warming, drought, nitrogen deposition and soil nutrient availability have all been shown to alter plant VOC emission patterns through reprogramming plant primary and secondary metabolism (Loreto et al. 2014). Consequently, not only will the VOC emission patterns of the focal plant be changed, but its background VOC emissions will also be changed, potentially challenging the plant in effectively communicating with its associated community members. Indeed, an ever-increasing number of studies have found various and far-reaching direct and indirect impacts of global change on VOC-mediated multitrophic interactions (Loreto et al. 2014; Blande et al. 2014). Unfortunately, we still know nothing about whether, and if so how, changes in VOC emissions would affect the efficacy of plant–plant communication. Does the altered ability of plants to emit VOCs affect the detectability of volatile cues by neighbouring plants, and do these global change factors alter the capability of the receiver plants to perceive and respond to volatile cues? Recent studies on the impacts of light conditions on VOC emissions exemplify how to address these questions and point out that changes in VOC emissions induced by environmental factors could potentially influence VOC-mediated plant–plant communication (Kegge et al. 2013, 2015; Pierik et al. 2014). The authors found that shading or far-red light enrichment that prevails at high plant densities reduced the quantity of VOCs and altered the VOC composition in both barley and *Arabidopsis*. Their follow-up test with barley demonstrated that reduced total VOC emissions from barley cultivar 'Alva' caused neighbouring cultivar 'Kara' to alter its carbon allocation (Kegge et al. 2015).

The context dependency of plant–plant chemical communication is further complicated by the as-yet unknown stability of VOCs under different environmental conditions. Once released into the air, VOCs begin to undergo dilution by turbulent diffusion, as well as chemical degradation by atmospheric oxidising agents. These include several anthropogenic pollutants such as ozone (O_3), hydroxyl radical (OH) and nitrogen oxide (NO_x) that react rapidly with many VOCs. As a result, the distance over which VOCs can travel depends not only on atmospheric conditions such as wind speed, air humidity and temperature but also on the presence of anthropogenic pollutants. While much is known about the impacts of VOC dilution in air currents on the efficacy of plant–plant communication, as shown by several field studies showing an effective signalling range of <1 m (Karban et al. 2000; Heil and Adame-Álvarez 2010), we are just beginning to understand the potential impacts that the presence of atmospheric oxidising agents, particularly those of anthropogenic origin, exerts on VOC-mediated plant–plant signalling (Blande et al. 2014; Li and Blande 2015). Laboratory and modelling studies have revealed that O_3- or NO_x-initiated VOC degradation may impair the efficiency of foraging by herbivores, parasitoids and pollinators (Pinto et al. 2007; Girling et al. 2013; Farré-Armengol et al. 2016) as well as the efficiency of plant–plant signalling (Blande et al. 2010; Li and Blande 2015). Atmospheric oxidising pollutants not only break down VOCs, the oxidation products may also nucleate in the atmosphere to form secondary organic aerosols. Adsorption of oxidation products and deposition of aerosol particles may have further ecological effects on plant–plant communication as well as plant–insect interactions. However, these aspects have not been assessed so far and merit future exploration.

7.5 Fitness Consequences of Plant Volatile Recognition

We know much less about the fitness consequences of plant–plant communication via VOCs than we do about the phenomena themselves. Most studies of plant responses to VOCs have documented physiological, biochemical or genetic changes in the receiver plants that would presumably convey herbivore resistance, as well as the changes in interactions among receiver plants, herbivores and predators or parasitoids. For instance, changes in physiological or phenotypic traits render the receiver plant less acceptable or palatable to herbivores and more attractive to parasitoids or predators. Comparatively, whether these changes will ultimately translate to fitness benefits in the receiver plants has been poorly explored. This has been most examined in three systems—wild tobacco (*Nicotiana attenuata*), wild sagebrush (*Artemisia tridentata*) and wild lima bean (*Phaseolus lunatus*) (Karban and Maron 2002; Kost and Heil 2006; Karban et al. 2012). Wild tobacco plants growing downwind from experimentally clipped sagebrush suffered less herbivory and produced as many or more flowers and seed capsules as those close to unclipped sagebrush neighbours (Karban and Maron 2002). A long-term field study on communication between sagebrush individuals showed similar results, with branches near clipped neighbours producing more inflorescences and seedlings being more likely to survive compared to those near unclipped neighbours (Karban et al. 2012). Similarly, native lima bean plants produced more flowers and shoots in response to volatile cues released from conspecific neighbours that had been experimentally damaged by herbivores (Kost and Heil 2006; Heil and Silva Bueno 2007). In contrast, a manipulative field survey with three annual species (*A. mollis*, *L. nanus* and *S. arvensis*) found no evidence in this regard (Pearse et al. 2012). Rather, this study showed that in all three species, damage to a neighbour decreased the lifetime seed production and fruit production of a genetically closely related receiver as well as other fitness measures, but did not affect the genetically distant receiver. Collectively, these studies suggest that the fitness outcome of responding to VOCs is species and context specific and determined by many biotic and abiotic factors. For instance, plant VOCs often serve as 'public' information available to any organism that can access them. As such, VOCs from damaged emitters may attract herbivores, which may in turn increase herbivory pressure on a neighbouring plant even without information exchange between them. Alternatively, responding to volatile cues indicating one type of stress may affect the plant's ability to respond to other types of stress.

The fitness consequences of plant–plant communication for emitter plants remain unclear. How can plant–plant communication be evolutionarily stable if the emitter advertises information that benefits its neighbours that could potentially compete for resources? Several explanations have been put forward (Heil and

Karban 2009). For example, plants do not emit VOCs intentionally to warn their neighbours, but to attract natural enemies of the attacking herbivores, to repel herbivores and/or to coordinate systemic responses in the remote parts of plants that are poorly connected vascularly to the damage site (Frost et al. 2008; Heil and Karban 2009). However, whether perception of signals by receiver plants and subsequent defence responses could have feedback effects on the emitters is not yet known. The finding that VOCs mediate kin recognition highlights that, through emitting VOCs, plants can increase inclusive fitness by warning their genetically related neighbours (Karban et al. 2013).

7.6 Future Research

While the concept of plant–plant communication via volatiles underwent a long period of debate, there is now convincing evidence that plant VOCs hold ecologically relevant information that neighbouring plants, particularly close relatives, can recognise and respond to. Nevertheless, there are still many questions that need to be answered before we can attain a more holistic understanding of the phenomenon and the mechanisms behind it. The predisposition of most researchers to report statistically significant or ‘positive’ results may have also hindered our understanding of this phenomenon. In order to further identify the signalling compounds that mediate plant–plant communication and the context in which information exchange between plants occurs, accurate reporting of experimental results, including negative results that do not conform to our current paradigms, is essential. Below, I outline some key areas where future efforts would offer great promise for discovery and even significant breakthroughs.

First, future research should continue characterising the bioactive volatile components in the VOC blends that reliably transmit information between plants, examining the specific qualitative and quantitative features of the compounds that plants perceive. Moreover, the interaction of different active compounds, the role of the whole VOC blend as well as the impact of background VOC noise also need to be evaluated. This knowledge is important as it can be exploited to tweak our current agricultural practices to make crops better defended against pests. For example, we can genetically modify plants to make them more sensitive to pest attack and more rapid at releasing key active compounds. When grown alongside field crops, these plants, like a ‘sentinel’, would be the first to detect and react to danger, alarming neighbouring crop plants.

Second, more efforts need to be diverted into understanding the recognition and perception of volatile cues as well as the further signal transduction. The big challenge is to determine whether these signals are internalised and transduced by receptor-mediated processes, whether they interact with the plasma membrane to initiate signal transduction cascades or whether they are simply taken up by the plant and metabolised into defensive compounds. The characterisation of receptors that are able to detect volatile compounds remains the biggest challenge.

Third, the ecological and evolutionary significance and the fitness consequences of information exchange between plants are still largely unexplored. More studies in a variety of habitats and using more plant species with different life histories are needed to determine the prevalence and ecological importance of plant–plant VOC transmission. Although plants are clearly responsive to volatile cues, it remains unclear how frequently or reliably plant VOCs transmit information between plants in natural settings. The lifetime fitness consequences for both the VOC emitters and responders require thorough scrutiny, particularly under natural conditions. This will help to elucidate the adaptive functions of VOC emissions and the driving force in the evolution of plant–plant volatile communication.

Finally, the impacts of global change on the efficacy of plant–plant VOC transmission need to be further evaluated. VOC emissions vary substantially in nature due to abiotic factors such as temperature, wind and radiation and will become more variable and unpredictable under local and global change. Oxidising agents released into the atmosphere due to human activities accelerate the oxidation of many VOCs reducing their lifespan in the atmosphere. These factors would either individually or collectively cause volatile cues that are reliable at the release point to lose reliability when they travel to the headspace of receiver plants. Furthermore, reaction products, including aerosol particles formed during chemical reactions, may also affect plant responses to volatile cues. In fact, the unpredictable stability of VOCs under global change is a crucial issue for estimating the true relevance of plant–plant volatile communication in natural ecosystems and for using plant VOCs as defensive weapons in biocontrol programmes.

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Chapter 8
Calling in the Dark: The Role of Volatiles for Communication in the Rhizosphere

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Abstract Volatile organic compounds play an important role in the communication between plants and other organisms. The rhizosphere contains a large and diverse microbial community whose members use similar volatiles for intra- and interspecific communication. However, the analysis of volatiles produced in the rhizosphere and their ecological functions have been little explored so far. In this chapter, we outline what is known about the classes of volatiles that are emitted into the rhizosphere by roots and soil microbes, and the effect they have on different interactors in the soil. Additionally, we review current approaches to sample volatiles in mesocosms and field soils. We conclude that to better understand the production and functions of volatiles in the rhizosphere, it is of critical importance to design set-ups that account for the natural complexity of soils. This will help to apply this knowledge for sustainable agriculture and the identification of novel agrochemicals.

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8.1 Introduction

The essential role of plant volatiles for communication with other organisms, or in other words as ‘infochemicals’, has been acknowledged for over 25 years (Dicke and Sabelis 1988). Nevertheless, their ecological functions have been mainly studied for aboveground interactions (e.g. Dicke and Baldwin 2010). However, it is well known that plant roots contain and produce similar classes of volatiles as aboveground organs. These volatiles are emitted especially in the rhizosphere. The rhizosphere, defined by Lorenz Hiltnet as the narrow zone surrounding and influenced by plant roots, is a hot spot for numerous organisms and is considered one of the most complex ecosystems on Earth. Organisms found in the rhizosphere include bacteria, fungi, oomycetes, nematodes, protozoa, algae, viruses, archaea, annelids and arthropods (Bonkowski et al. 2009; Buee et al. 2009; Raaijmakers et al. 2009).

Most members of the rhizosphere community are part of a complex food web that utilises the large amount of nutrients released by the plant. Rhizosphere organisms that have been well studied for their beneficial effects on plant growth and health include the nitrogen-fixing bacteria, mycorrhizal fungi, plant growth-promoting rhizobacteria (PGPR), mycoparasitic fungi and protozoa. For example, 80 % of terrestrial plant species actively associate with mycorrhizal fungi that may help the plant to overcome nutrient limitations in exchange for carbon resources (van der Heijden et al. 2015). In recent years, primarily driven by efforts towards sustainable intensification in agriculture, there has been an increased interest in PGPR. The main benefit of PGPR can be ascribed to direct growth promotion or to indirect effects via the protection of plants against (a) biotic stresses (Bulgarelli et al. 2012). Decomposers in particular, ranging from small organisms such as bacteria, fungi or nematodes to large macrofaunal organisms such as earthworms and dung beetles, are essential elements of the soil food web. They ensure that dead plant materials re-enter the soil nutrient cycle, thereby increasing plant growth (Kulmatiski et al. 2014).

Even though decomposers certainly may have an effect on, or be affected by, plant volatiles in the rhizosphere, in this chapter, we will focus mainly on the role of volatiles in communication between plants and rhizosphere organisms interacting with living roots. This also includes communication with organisms functioning at higher trophic levels, such as parasitoids or pathogens of root-feeding organisms. Rhizosphere organisms that are deleterious to plant growth and health include pathogenic fungi, oomycetes, bacteria, nematodes and insect herbivores (Blossey and Hunt-Joshi 2003; Bonkowski et al. 2009; Kulmatiski et al. 2014; Mendes et al. 2013). Despite their small size, soil pathogens can cause substantial agricultural losses and are also involved in large-scale ecosystem processes such as succession (de Deyn et al. 2003).

For each organism on earth, it is important to obtain information on the quality of its environment in order to assess opportunities and dangers. Aboveground, vision and light sensing play an important role for both autotrophic as well as heterotrophic organisms (Döring 2014; Kegge et al. 2015). However, belowground, this option is lacking due to the absence of sunlight, which makes chemical communication the more likely way for interaction partners to localise and recognise each other (van Dam 2009). For example, to establish their intimate relationship, host plants and mycorrhizal fungi exchange elaborate chemical communication involving non-volatile strigolactones produced by plant roots and lipochitooligosaccharides produced by arbuscular mycorrhizal fungi (reviewed in van der Heijden et al. 2015). Similarly, rhizosphere bacteria communicate with each other using, e.g. *N*-acyl homoserine lactones (AHL) in a process called 'quorum sensing' to assess if there is enough critical mass to colonise a plant or to form a biofilm (Bakker et al. 2013). This type of communication between collaborating partners can be tapped into by others. For example, parasitic plants use strigolactones to locate their host plant and optimise their timing of germination (Cardoso et al. 2011). Plants that perceive the increase in AHL in their rhizosphere may interfere with this bacterial communication by producing AHL mimics (Teplitski et al. 2000).

Since the early years of the twenty-first century, it has become increasingly clear that plant-produced volatile organic compounds such as terpenoids are also actively involved in rhizosphere communication (Rasmann et al. 2005; van Tol et al. 2001). Initially, these findings were met with scepticism; non-polar volatiles such as terpenoids were not considered to have the optimal chemical properties for travelling in a humid and dense medium such as the soil. This scepticism was experimentally refuted by the fact that the sesquiterpene (*E*)-β-caryophyllene, which is emitted by maize roots, diffuses best in the gaseous phase of humid soils (Hiltpold and Turlings 2008). At the same time, volatiles may be more stable and reliable cues for communication belowground than in the air, because of the lack of UV-light and the relatively constant temperature in the soil. Hence volatiles may be excellent vehicles to communicate between organisms in the rhizosphere.

In this chapter, we first outline which classes of volatiles are produced by the different organisms in the rhizosphere. Here we will focus on the production of volatiles by roots and microorganisms. It is very possible that other soil-dwelling organisms, such as insects and nematodes, also produce volatiles, but evidence to support this is currently lacking. Second we will outline what is known about the ecological roles of the different volatiles produced by plants and microbes in communication between different members of the soil community. Then we will review the various approaches that are currently used to sample and analyse rhizosphere and root-emitted volatiles. In our conclusion, we discuss the potential of certain volatiles to be the 'lingua franca' for communication between different taxa whose members interact in the rhizosphere. Moreover, we will discuss how the distinct roles of specific volatiles can be assessed experimentally and how we can explore their effect in belowground interactions.

8.2 Production of Volatiles in the Rhizosphere

8.2.1 Microbes in the Rhizosphere

Plant scientists frequently perceive plants as relatively independent organisms that rely on soil mineral nutrients, water and sunlight, while the role of microbes in plant life is restricted to that of pathogenic microbes or a few well-characterised symbionts, such as nitrogen-fixing bacteria. However, plants are colonised by an astonishing number of microorganisms, whose numbers supersede the number of plant cells. Moreover, the number of microbial genes in the plant rhizosphere by far outnumbers that of plant genes (Mendes et al. 2013). Most studies to date have mainly focused on the number and diversity of bacterial taxa in the rhizosphere, and depending on the sequencing techniques used, the reported numbers range from <100 to more than 55,000 operational taxonomic units (OTUs). Most rhizospheres are dominated by *Proteobacteria*, *Bacteroides*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia* or *Firmicutes* (Badri et al. 2009; Berendsen et al. 2012; Bulgarelli et al. 2012; Mendes et al. 2013). Within the group of *Proteobacteria*, in addition to well-studied and described Gamma-*Proteobacteria* (*Pseudomonas*) and Alpha-*Proteobacteria* (*Rhizobia*), the importance of Beta-*Proteobacteria* (*Oxalobacteraceae* or *Burkholderia*) is increasingly recognised, due to their high relative observed abundance in rhizosphere metagenome surveys.

For a long time, it has been assumed that the rhizosphere is mainly dominated by bacteria, as fungi are mostly known to be involved in the decomposition of recalcitrant soil organic matter (de Boer et al. 2006). However, recent studies revealed significant utilisation of root exudates by saprotrophic fungi (Buee et al. 2009). These can either be fungi that can co-metabolise root exudates while decomposing recalcitrant organic matter or fungi that are specialised to decompose simple metabolites such as mono- and disaccharides, the so-called ‘sugar fungi’ (Buee et al. 2009). In addition, pre-infective growth of plant pathogenic soil fungi is also dependent on the availability of root exudates (Njoroge et al. 2008). Microorganisms living in the rhizosphere interact with plants in many ways and can have profound effects on plant growth and development by different plant growth-promoting mechanisms such as nitrogen fixation, phytohormone production, induction of systemic resistance or inhibition of phytopathogenic fungi (Berendsen et al. 2012; Lugtenberg et al. 2001; Mendes et al. 2013). Recent studies have revealed that the production of volatile organic compounds by plant-associated microorganisms can play a major role in long-distance plant–microbe interactions.

8.2.2 Volatiles Produced by Microbes

Microbial volatile compounds are produced by a wide array of microorganisms including bacteria and fungi. Most microbial volatiles are considered as by-products of primary and secondary metabolism. They are formed mainly by oxidation of glucose from various intermediates (Korpi et al. 2009). The underlying biosynthetic pathways are aerobic metabolism, heterotrophic carbon metabolism, fermentation, amino acid catabolism, terpenoid biosynthesis, fatty acid degradation and sulphur reduction (Peñuelas et al. 2014a). Recently, a microbial volatile organic compounds database, mVOC (http://bioinformatics.charite.de/mvoc), was developed where all microbial volatiles reported to date are compiled. This database reveals that bacterial volatiles are dominated by (in descending order) alkenes, alcohols, ketones, terpenes, benzenoids, pyrazines, acids and esters, whereas fungal volatile profiles are dominated by alcohols, benzenoids, aldehydes, alkenes, acids, esters and ketones. Below, we briefly review the biosynthesis of the most prominent volatile classes produced by microbes, which will later be compared with volatile production in plants.

Aromatic compounds are generated in bacteria and fungi via the shikimic acid pathway. 2-Phenylethanol, which is one of the most commonly emitted volatile aromatic compounds, is synthesised by using L-phenylalanine as a precursor. An aminotransferase catalyses the transamination to phenylpyruvate, followed by an oxidative decarboxylation to phenyl-acetaldehyde and an NADH-dependent reduction to the corresponding alcohol (Hazelwood et al. 2008; Kim et al. 2014).

Many bacterial and fungal volatile blends contain **aliphatic hydrocarbons**, mainly alkenes, alcohols and ketones. These compounds are typically derived from fatty acids, which are synthesised from acetyl-CoA via conversion into malonyl-CoA (Jenni et al. 2007; Schulz and Dickschat 2007).

Terpenoids represent one of the largest classes of volatiles with over 50,000 known members. Although they are mostly known as plant metabolites, it recently has become clear that microorganisms are a rich source of terpenes (Dickschat et al. 2014). An increasing number of terpenes has been reported for several soil-derived fungi, most of them being sesquiterpenes (Collado et al. 2007; Ebel 2010; Singh et al. 2011). One of the most well-known microbial volatiles is geosmin, a sesquiterpenoid responsible for the characteristic earthy odour of moist soil. Despite their remarkable chemical and functional diversity, the biosynthesis of all terpenoids starts from just a few acyclic precursors, including prenyldiphosphate, geranyl diphosphate (GPP, C10), farnesyl diphosphate (FPP, C15) and geranylgeranyl diphosphate (GGPP, C20) (Dickschat et al. 2014). Terpene synthases are the primary enzymes responsible for catalysing the formation of hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15) or diterpenes (C20) from the substrates DMAPP, GPP, FPP or GGPP, respectively (Tholl 2006). The recently increased knowledge about bacterial genomes revealed many distinct terpene synthase genes widely distributed in bacteria, indicating that bacteria can be a rich source of terpenes (Cane and Ikeda 2012; Yamada et al. 2012, 2015). Many soil- and plant-associated bacteria harbour genes encoding such terpene synthases. However, most of these genes are silent in the parent microorganisms under laboratory culture conditions and only for few bacterial strains have the terpene synthases been chemically characterised. Although the principal processes of terpene biochemistry are well understood, it is difficult to predict terpene structures from the amino acid sequence of terpene synthases. To date, studies on bacterial terpenes were done mostly on *Streptomyces* spp. and only one terpene cyclase from *Proteobacteria* has been functionally characterised, the 2-methylenebornane synthase from *Pseudomonas fluorescens* Pf0-1 (Chou et al. 2011).

Recently, it was found that *Collimonas* strains (belonging to the class of Beta-*Proteobacteria*) harbour terpene synthase genes (CPter91_2617 and CPter291_2730; Song et al., 2015). When compared to other functionally characterised terpene cyclases, the *Collimonas* protein sequences showed maximally 23 % aa-identity to any previously characterised bacterial terpene cyclase. As the product specificity of mono- and sesquiterpene cyclases cannot be predicted from their primary biochemical characterisation, CPter91_2617 and CPter291_2730 genes were expressed in *E. coli* and tested for cyclization reactions using FPP, GPP or GGPP as substrates. When produced terpenes were analysed by gas chromatography–mass spectrometry (GC-MS), both *Collimonas* enzymes converted FPP to a mix of sesquiterpenes and sesquiterpene alcohols. The major peak was putatively identified as germacrene D-4-ol by comparison of the mass spectrum to a spectral library, as well as several minor sesquiterpene peaks which included δ-cadinene. When GPP was applied as a substrate, the production of two monoterpenes identified as β-pinene and β-linalool was observed (Song et al., 2015). The sesquiterpene products suggest that they are functionally related to plant and fungal cadinene/cadinol and germacrene D-4-ol synthases (Lauchli et al. 2014; Yoshikuni et al. 2006).

Volatile **sulphur compounds** play central roles in global sulphur biogeochemical cycles (Naeem 1998). The structural diversity of these compounds is large, ranging from relatively small compounds such as methanethiol, dimethyl sulphide (DMS), dimethyl disulphide (DMDS) and dimethyl trisulphide (DMTS) to more complex volatiles, such as 2-methyl-4,5-dihydrothiophene (Effmert et al. 2012; Splivallo et al. 2011). Two main biosynthetic pathways, both relying on L-methionine catabolism, have been described: the one-step conversion of L-methionine to methanethiol by methionine c-lyase or by other C-S lyases (e.g. cystathionine c-lyase) and a two-step pathway, initiated by L-methionine transamination to 4-methylthio- 2-oxobutyric acid, which is then converted to 3-(methylthio)propanal via decarboxylation. Alternatively, L-methionine is reduced to 4-methylthio-2-hydroxybutyric acid which ultimately results in the formation of methanethiol (Splivallo et al. 2011). DMS emission requires the gene *dddD* which was predicted to add CoA to dimethylsulphoniopropionate (DMSP), a key step preceding subsequent cleavage and release of DMS (Todd et al. (2007). DMS is mostly made via bacterial catabolism of dimethylsulphoniopropionate (DMDP). This so-called Ddd^{+} trait is found in several genera belonging to the phylum *Proteobacteria* (Peng et al. 2012; Todd et al. 2011, 2012). Microbial sulphur volatile compounds such as DMS, DMDS and DMTS play important roles in plant–microbe and interspecific fungal–bacterial and bacterial–bacterial interactions (see below).

8.2.3 Volatiles Emitted by Plant Roots

Chemical analyses of essential oil extracts show that roots are a rich source of plant volatiles. For example, vetiver grass (*Chrysopogon zizanioides*) root extracts, which are traditionally used in the perfume industry, may contain up to 300 different volatile compounds (Belhassen et al. 2015). However, whether these volatiles are emitted in the rhizosphere in vivo and in the same ratios as they are present in the root is as yet unknown (Peñuelas et al. 2014a, but see Jassbi et al. 2010). Thus instead of listing all possible volatile compounds that have been identified in roots and root extracts, we mainly focus on volatiles that have been shown to be emitted by roots into the rhizosphere or the root headspace as measured by non-destructive sampling techniques.

Small Organic Volatiles Carbon dioxide (CO_2) is one of the smallest volatiles that roots excrete as a result of their own respiration (Ghashghaie and Badeck 2014). In addition, plant roots may emit various alcohols, ketones and esters, such as methanol, acetone and ethyl acetate (Danner et al. 2015; Steeghs et al. 2004). These small organic volatiles are considered to be by-products of the plant's primary processes. For example, the production of methanol is correlated with the activity of methylesterases involved in the loosening of cell walls which allows root growth and the release of root border cells (Driouich et al. 2013). Furthermore, aldehydes and short-chain fatty acid-derived C6 volatiles, such as hexanal and hex-2-en-1-ol, have also been detected in the root headspace (Peñuelas et al. 2014a; Steeghs et al. 2004). These compounds are produced from fatty acids such as linoleic or linolenic acid, which serve as substrates to 13-lipoxygenases (LOX). Plants contain several different LOX enzymes allocated to different plant organs including the roots, and with different functions in the response to abiotic and biotic stress signalling (Allmann et al. 2010; Grebner et al. 2013).

Terpenoids Similar to their biosynthesis by microbes, the synthesis of terpenoids in plants may take place via the precursors DMAPP, GPP, FPP or GGPP. In plants, however, terpenoid synthesis can either take place via the mevalonic acid (MVA) or the methylerythritol 4-phosphate (MEP) pathways. In plant cells, these two pathways are compartmentalised; the MEP pathway is localised in the plastids, whereas the enzymes of the MVA pathway are localised in the cytosol (Gutensohn et al. 2013). Interestingly, this separation may be a remnant of evolution past when ancient eukaryotes engulfed cyanobacteria to form a symbiotic complex that evolved into higher plants (Wiesner et al. 2013). It is known that there is some cross-talk between the two biosynthetic pathways, but it is still generally assumed that monoterpenes (C10), diterpenes (C20) and more complex terpenoids, such as gibberellins and chlorophylls, are mainly produced in the plastid via the MEP pathway. Sesquiterpenes (C15), sterols and triterpenes (C30) are mainly produced in the cytosol via the MVA pathway (Gutensohn et al. 2013; Harrison et al. 2013; Peñuelas and Munne-Bosch 2005). Even though this knowledge is mainly based on studies analysing the biosynthesis of flower and leaf terpenoids, it seems that the subcellular localisation of the MEP pathway in root cells is similarly arranged. Genes involved in root-specific mono- and diterpene synthesis in the model plant *Arabidopsis thaliana* were found to have motifs that predestine them for plastid targeting (Chen et al. 2004; Vaughan et al. 2013). In maize, the root-specific gene farnesyl diphosphate synthase (fpps1) involved in herbivore-induced synthesis of the sesquiterpene (*E*)-β-caryophyllene indeed appeared to be located in the cytosol (Richter et al. 2015). Emissions of terpenes from the roots can strongly increase upon damage by insect herbivores. This is not only due to passive release of terpenoids from the wounds but involves active expression of terpene synthases (TPS) in the root tissue as well as de novo synthesis of terpenoids (Chen et al. 2004; Rasmann et al. 2005; Richter et al. 2015).

Sulphur- and Nitrogen-Containing Compounds As well as C-based terpenoids, plants may produce a range of sulphur- and/or nitrogen-containing volatiles. Some of these volatiles are produced by special prefabricated two-component systems consisting of a glycosylated precursor compound and a β-glucosidase. Enzymes belonging to this class catalyse the hydrolysis of a β-glucosidic bond between two carbon moieties or between a carbohydrate and an aglucone moiety (Morant et al. 2008). This reaction results in the release of an aglucone, which may be further converted in bioactive volatiles, especially in the case of cyanogenic glycosides and glucosinolates (Kissen et al. 2009). In cassava roots, for example, cyanogenic glycosides stored in the vacuole react with β-glucosidases upon tissue rupture. This leads to the production of an unstable aglucone, which spontaneously degrades into the highly toxic volatile HCN. Cyanogenesis is a widespread trait and has been found to occur in more than 2600 plant species ranging from gymnosperms to mono- and dicotyledonous species (Morant et al. 2008).

A similar two-component system yielding sulphur- and nitrogen-containing volatiles is found in Brassicaceae. Members of this plant family contain sulphur-containing defence compounds, called glucosinolates. Upon tissue rupture, the glucosinolates in the vacuoles come into contact with myrosinase, a glucosidase that is stored in specialised cells (Bones and Rossiter 2006). As a consequence, sulphur- and/or nitrogen-containing volatiles, such as isothiocyanates (ITCs) and nitriles, are formed. These sulphur- and nitrogen-containing volatiles may serve different functions, among others as defences against insect herbivores, nematodes and (soil) pathogens (Brown and Morra 1997; Caboni et al. 2012; Hopkins et al. 2009). Overall, more than 130 structurally different glucosinolates have been identified to date (Agerbirk and Olsen 2012), and their chemical structure, together with the presence or absence of nitrile-specifier enzymes and the pH at the site of the reaction, greatly determines the types of volatiles that are formed (Halkier and Gershenzon 2006). Interestingly, overall root glucosinolate concentrations are higher than those in shoots, and specific glucosinolates, such as 2-phenylethyl glucosinolate or 1-methoxy-indol-3-ylmethyl glucosinolate (neoglucobrassicin), are more prominent in belowground organs (van Dam et al. 2009). This suggests a specific role for the volatile products that are formed in the rhizosphere. Indeed, 2-phenylethyl ITC was shown to confer resistance to root-feeding nematodes and soilborne pathogens (Potter et al. 1998; Sarwar et al. 1998).

In addition to ITCs, roots of *Brassica* species may produce a range of other sulphur-containing volatile compounds such as methanethiol, DMS, DMDS and DMTS (Crespo et al. 2012). Depending on the species, their emissions may increase upon root herbivory or mechanical wounding (van Dam et al. 2012). In plants, these sulphides may either result from thiol methyltransferases involved in the catabolism of glucosinolate conversion products, possibly to avoid autotoxicity (Attieh et al. 2000), or a combination of cysteine-sulphoxide lyases involved in the final degradation steps of the nonprotein amino acid *S*-methyl-L-cysteine (Chin and Lindsay 1994). Both ITC and sulphides from *Brassica* roots are found to be emitted constitutively at low levels, probably due to some spontaneous or chemically driven degradation of the precursor glucosinolate or to the continuous turnover of root tips (Bones and Rossiter 2006).

Another class of well-studied sulphur-containing rhizosphere volatiles produced by plant roots are thiophenes. These compounds are produced in the roots of Asteraceae, especially species of the genus *Tagetes* (Marigolds) (Croes et al. 1989; Jacobs et al. 1994). Thiophenes are well known for their nematicidal, antimicrobial and insecticidal effects, though soil microbial communities as a whole do not seem to be affected by marigold cultures (Caboni et al. 2012; Leger and Riga 2009). In situ analyses using passive sampling approaches combined with GC-MS analyses have shown that thiophenes are constitutively emitted into the rhizosphere by *Tagetes* roots (Mohney et al. 2009; Tang et al. 1987). *Tagetes* roots contain specialised structures, such as secretory channels in the root endodermis, which would allow a constant emission of thiophenes into the rhizosphere (Sacchetti et al. 2001).

Volatile Phytohormones Several volatile signalling hormones are emitted by roots. Ethylene is by far the most studied volatile plant hormone. 1-Aminocyclopropane–carboxylic acid (ACC) is the direct precursor of ethylene and is synthesised from methionine. The enzyme ACC oxidase catalyses the final step in the synthesis of ethylene (Gepstein and Kieber 2010). It serves as a signalling hormone involved in biotic and abiotic stress responses, including shade avoidance, leaf senescence and the formation of root hairs (Gepstein and Kieber 2010; Pierik et al. 2006). Maize root systems constitutively emit ethylene, which is reduced when the plants are infested either aboveground or belowground by herbivores (Robert et al. 2012). Abiotic stresses, such as waterlogging, may enhance ethylene emissions by roots. For example, ethylene accumulates in *Solanum dulcamara* plants subjected to water logging, leading to the formation of aerenchymous adventitious roots that facilitate gas exchange underwater (Dawood et al. 2014).

Methyl salicylate (MeSA) is the volatile methylated form of the phytohormone salicylic acid (SA), which is produced via the shikimic acid pathway. The enzyme *S*-adenosyl-L-methionine:salicylic acid carboxyl methyltransferase (SAMT) converts SA into volatile MeSA (Dudareva et al. 2004). In aboveground plant organs, MeSA is involved in responses to biotrophic pathogens and piercing–sucking herbivores (De Vos et al. 2005). Aboveground MeSA is often induced by herbivore feeding and consequently is used by natural enemies as a cue to localise their host (De Boer et al. 2004; Kpoviessi et al. 2011). There is only indirect evidence that MeSA may play a similar role belowground. Roots of poplar trees, for example, contain a methyltransferase with high homology to SAMT that is able to convert SA into MeSA (Zhao et al. 2009). In leaves, the expression of this methyltransferase was strongly induced by wounding, SA and methyl jasmonate (MeJA) application; however, this was not tested for expression in roots (Zhao et al. 2009). In hairy root cultures of *Atropa belladonna*, SAMT activity was increased when the cultures were induced with SA (Fukami et al. 2002).

Taken together, these results suggest that methyltransferase gene activity and the production of MeSA by roots may indeed play a role in rhizosphere communication (Fukami et al. 2002; Loreto and Schnitzler 2010; Zhao et al. 2009). However, direct evidence that MeSA is emitted in the rhizosphere is lacking. Similarly, reports on the emission of MeJA in the headspace are scarce. *Artemisia tridentata* roots were found not to emit MeJA, even though it is one of the most prominent volatiles produced by the shoots (Jassbi et al. 2010). According to a recent review, the necessary enzymes are not found in roots, despite the presence of the LOX pathway and the fact that roots respond well to MeJA treatments (Peñuelas et al. 2014a).

8.3 The Ecological Role of Volatiles in the Rhizosphere

Volatiles play a versatile role in communication between the various members of the soil community. The interactions they are involved in and the volatiles that have been identified as critical actors are summarised in Fig. 8.1 and discussed in the following sections.

8.3.1 Microbial Volatiles and Their Effect on Fungi and Oomycetes

Although the importance of volatiles as major fungistatic compounds has long been recognised (Hora and Baker 1970, 1972), this topic has received more extensive research attention in recent years. Surveys of soil bacteria have reported that 30–60 % of soil isolates can produce fungus-inhibiting volatiles (Wheatley 2002; Zou et al. 2007), and that these organisms span a wide phylogenetic spectrum, including members of the *Alcaligenaceae*, *Bacillales*, *Burkholderia*, *Collimonas*, *Micrococcaceae*, *Pseudomonas*, *Rhizobiaceae*, *Serratia*, *Xanthomonadaceae* and many others (Blom et al. 2011a; Effmert et al. 2012; Kai et al. 2007; Zou et al. 2007). Compared to the plant response to bacterial volatiles, which has almost exclusively been restricted to *Arabidopsis*, assays investigating the response of fungi to bacterial volatiles have tested a broad range of phytopathogenic fungi and oomycetes (Effmert et al. 2012; Garbeva et al. 2014b; Kai et al. 2007). Germination

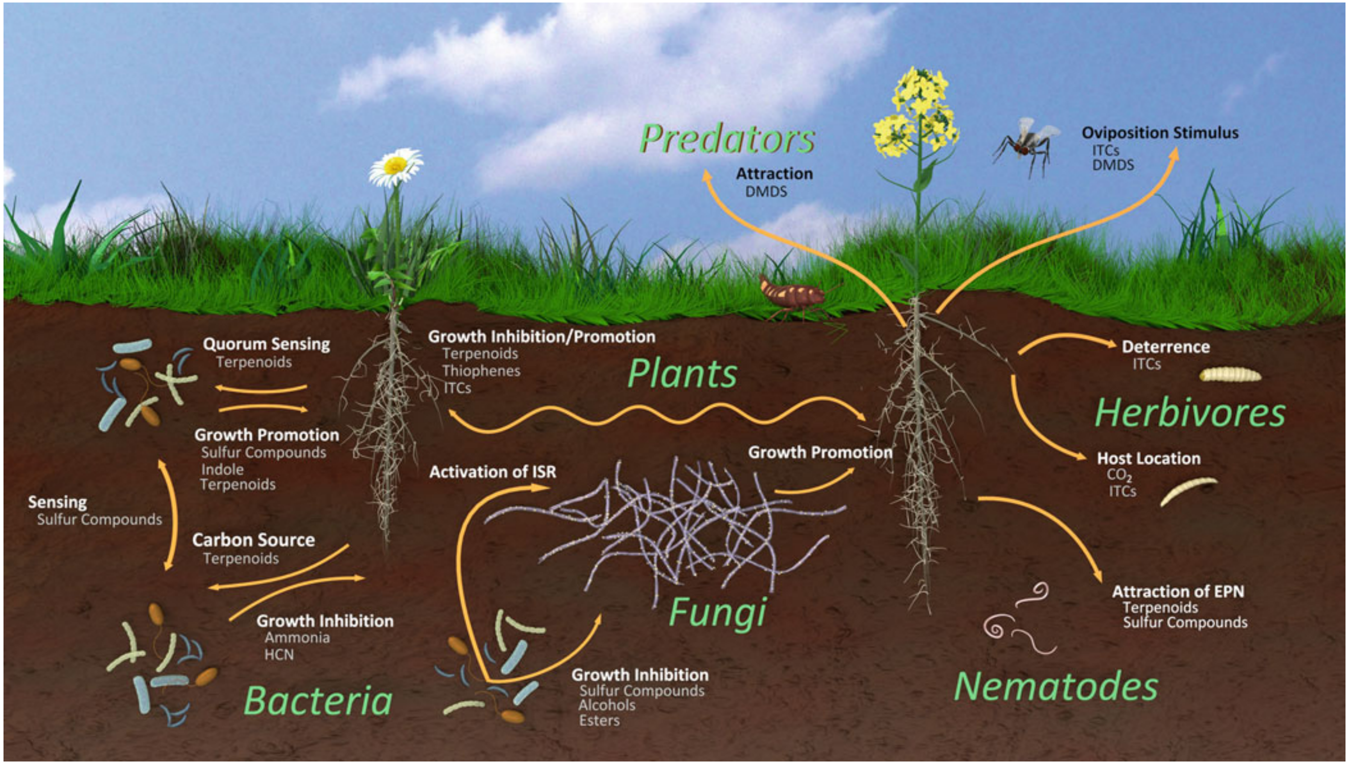

Fig. 8.1 Schematic overview illustrating the versatile roles of soilborne volatiles as currently documented in the scientific literature. Volatiles are released by the different groups of organisms in the rhizosphere, i.e. plant roots, bacteria and fungi. The direction of the volatile emission is indicated by *arrows* that point towards the receivers, which also include predators, insects and nematodes. Next to the *arrow*, the effect on the receiver is stated. The compound classes reported in the literature to mediate these effects are specified. Abbreviations: *DMDS* dimethyl disulphide, *HCN* hydrogen cyanide, *ITCs* isothiocyanates. Design: Alexander Weinhold, iDiv and Kimberly Falk (moveslikenature.com)

of fungal spores and hyphal growth can be strongly inhibited by bacterial volatiles. Furthermore exposure to bacterial volatiles has been reported to change fungal morphology, enzyme activity and gene expression (Garbeva et al. 2014b; Kai et al. 2008; Vespermann et al. 2007). Most work on microbial volatiles to date is done in vitro under nutrient-rich conditions (Kai et al. 2010; Weise et al. 2012) and may not be representative for the conditions that prevail in the natural environment. Recently Garbeva et al. (2014b) revealed that volatile production by *Collimonas* sp. in sand containing artificial root exudates differs from that on 1/10 TSBA agar plates. More than 45 % of the volatiles released by *Collimonas* on 1/10 TSBA were sulphur-containing volatiles, whereas the majority of volatiles released from the root exudate containing sand were ketones, aromatic volatiles and esters (Garbeva et al. 2014b).

Studies have tested many compounds individually over very different concentration ranges and with varying application modes. Some volatiles repeatedly showed inhibitory effects, including hydrogen cyanide (HCN), DMDS, DMTS, benzothiazole, benzaldehyde, benzonitrile and 2-undecanone (Fig. 8.1; Effmert et al. 2012; Garbeva et al. 2014b; Weisskopf et al. 2011). Hydrogen cyanide produced by some *Pseudomonas* species (such as *P. fluorescens* CHA0) was confirmed to be directly involved in the biocontrol of *Thielaviopis*-induced root rot of tobacco (Voisard et al. 1989). Application of DMDS produced by *Bacillus cereus* strains significantly protected tobacco plants against *Botrytis cinerea* and maize against *Cochliobolus heterostrophus* (Huang et al. 2012). Fungal volatiles can also have inhibitory effects on other fungi. For example, the endophytic fungi *Muscodor albus* and *Oxyporus latemarginatus* strongly inhibited growth of several plant pathogenic fungi including *Botrytis cinerea* and *Rhizoctonia solani* (Strobel et al. 2001).

Several independent studies have observed high variation in fungal sensitivity to bacterial volatiles (Effmert et al. 2012; Garbeva et al. 2014b; Kai et al. 2007, 2008; Weisskopf et al. 2011). For example, testing a range of saprotrophic and plant pathogenic fungi, it was revealed that *Rhizoctonia solani*, *Fusarium culmorum* and *Pythium ultimum* were the most sensitive, while the saprotrophic fungi *Chaetomium* sp., *Mucor hiemalis* and *Trichoderma harzianum* were the most resistant (Garbeva et al. 2014b). This confirms previous reports on difference in fungistasis sensitivity between pathogenic and saprotrophic fungi (Garbeva et al. 2011).

8.3.2 *Microbial Volatiles Produced as a Result of Interactions with Other Microbes*

Recently, several independent studies have reported that the production of specialised metabolites by soil bacteria is the direct result of interactions with other microorganisms in their immediate vicinity (Traxler et al. 2013; Tyc et al. 2014). This explains the fact that the genomes of soil and rhizosphere bacteria contain numerous cryptic gene clusters encoding genes involved in the production of secondary metabolites that are not expressed during growth under typical laboratory conditions.

Some volatiles appear to be emitted as result of microbial interactions. Recently, Minerdi et al. (2008) observed that antagonistic interactions between two *Fusarium oxysporum* strains were related to volatile production. Interestingly, the inhibiting volatiles were only produced when the antagonistic strain was associated with a consortium of bacterial species. The origin of the volatiles, i.e. bacterial or fungal, remained unclear, but the need for the bacterial–fungal association was evident (Minerdi et al. 2008). Furthermore, the composition of volatiles produced by a mixture of bacterial species can differ from those produced by each bacterial monoculture (Garbeva et al. 2014a). Recently Hol et al. (2015) revealed that less abundant (so-called 'rare') bacterial species play an important role in antifungal volatile production. The loss of rare soil bacteria affected the production of antifungal volatiles, an important factor in the natural control of soilborne pathogenic fungi (Hol et al. 2015). Furthermore, small shifts in soil microbial community composition can lead to significant shifts in volatile compositions (Schulz-Bohm et al. 2015). Microbial volatiles play important roles in the rhizosphere as infochemicals affecting the behaviour, populations and gene expression of responding organisms. For example, bacterial volatiles play an important regulatory role in mycorrhizal network establishment (Bonfante and Anca 2009) and volatiles from mycorrhiza helper bacteria (MHB) can promote the growth of ectomycorrhizal fungi (Schrey et al. 2005).

To obtain insight into the importance of interspecific volatile interactions between soil bacteria, Garbeva et al. (2014a) performed several microcosm experiments mimicking the natural nutritional heterogeneity in soil in which the model bacteria *P. fluorescens* grown on nutrient-limited agar was exposed to volatiles produced by four phylogenetically different soil bacteria growing in sand containing artificial root exudates. The main research questions addressed were: (1) Do rhizobacteria protect their 'territory' from potential rhizosphere invaders by producing volatiles that suppress bacteria outside the rhizosphere? (2) Can bacteria outside the rhizosphere profit from the volatiles produced by rhizosphere-inhabiting bacteria? Their results revealed that bacterial volatiles stimulated rather than inhibited the growth of *P. fluorescens*. A genome-wide microarray-based analysis revealed that exposure to bacterial volatiles had clear effects on gene expression in *P. fluorescens* and that the change in gene expression differed among the different volatile-producing bacterial species. Besides other transcriptional changes, such as those assigned to energy production and conversion, bacterial volatiles appeared to induce a chemotactic motility response in *P. fluorescens* but also an oxidative stress response. A more detailed study revealed that some of the volatile-producing bacteria triggered antimicrobial secondary metabolite production in *P. fluorescens* (Garbeva et al. 2014b). The volatile-triggered antibiotic production in *P. fluorescens* pointed to a strategy to combine movement (chemotaxis and motility genes) with increasing competitive strength (antibiotics) to invade into the nutrient-providing rhizosphere zone.

Volatiles may also be involved in tritrophic interactions involving bacteria, fungi and nematodes as shown by the work of Son et al. (2009). *Paenibacillus polymyxa* and *P. lentimorbus* exhibited strong antifungal activities, interfering with interactions between the nematode *Meloidogyne incognita* and the fungus *Fusarium oxysporum* which significantly reduced nematode infestation of tomato plants (Son et al. 2009). Recently it was reported that bacterial volatiles may interfere with the quorum sensing of other phylogenetically different bacteria due to suppression of the transcription of AHL synthase genes (Chernin et al. 2011). DMDS was identified as one such quorum sensing inhibiting compound (Fig. 8.1; Chernin et al. 2011). The same compound was reported to stimulate bacterial growth, whereas it completely inhibits fungal growth (Garbeva et al. 2014a, b; Kai et al. 2007).

8.3.3 Effect of Microbial Volatiles on Plants

Over the last decade, evidence has accumulated that plants respond strongly to volatiles produced by microorganisms. Most of the research carried out so far has investigated the impact of bacterial volatiles on the model plant *A. thaliana*. This has revealed that, without physical contact, bacteria are able to drastically alter the plant's root system development and biomass production, ranging from plant death to a sixfold increase in biomass compared with nonexposed plants. Significant growth promotion of *A. thaliana* after exposure to complex blends of volatiles emitted by a range of PGPR was reported by Ryu et al. (2003). Using two-compartment Petri dishes where only volatiles can be exchanged between the plant and bacteria, the authors obtained a fourfold growth promotion with two *Bacillus* strains. The growth promotion effect was ascribed to 2,3-butanediol and acetone, based on the application of pure compounds and based on lack of plant growth promotion after exposure to the volatiles emitted by a strain mutated in the butanediol fermentation pathway (Fig. 8.1; Ryu et al. 2003).

The opposite effect of bacterial volatiles on *A. thaliana* was reported by Vespermann et al. (2007), where plants exposed to volatiles emitted by *Serratia* strains were killed within a very short time. The effect was alleviated by addition of charcoal, demonstrating that the killing effects were indeed caused by the emitted bacterial volatiles (Vespermann et al. 2007). More recently Blom and coworkers assessed 42 bacterial strains originating from the soil and rhizosphere for emission of plant growth-modulating volatiles (Blom et al. 2011a, b). All strains were found to emit plant growth-modulating volatiles but with contrasting effects that strongly depended on the growth conditions. Dose-dependent plant growth-promoting effects were observed for several compounds including indole, 1-hexanole and pentadecane. For example, indole was active when applied in very low amounts and toxic when plants were exposed to higher amounts (e.g. 10 μg), while pentadecane was active when applied in high amounts (1 mg).

To understand plant physiological changes caused by exposure to bacterial volatiles, Zhang et al. (2007) applied a microarray approach to analyse genes expressed upon exposure to volatiles emitted by *Bacillus subtilis* GB03. The transcriptomic analysis revealed differential expression in about 600 genes, with auxin-related genes being particularly affected. Auxin synthesis appeared to be specifically increased in the aerial parts of the plants, but the auxins were actively transported as evidenced by a shift in auxin distribution from the shoots to the roots in response to volatile exposure (Zhang et al. 2007). Genes upregulated by exposure to volatiles emitted by *Bacillus subtilis* GB03 included ethylene biosynthesis and ethylene response genes, which were further confirmed at the proteome level by Kwon et al. (2010). Plant iron uptake can be increased by exposure to bacterial volatiles. This is linked to the acidifying potential of the produced volatiles, leading to better solubilisation and uptake of iron (Zhang et al. 2009).

One of the few identified volatile compounds showing growth promotion in *A. thaliana* is indole (Fig. 8.1; Blom et al. 2011a, b). Indole, a hetero-aromatic compound derived from L-tryptophan is emitted by a range of bacteria including PGPR *Pseudomonas* and *Burkholderia* (Audrain et al. 2015; Blom et al. 2011a, b; Zamioudis et al. 2013). Indole-producing bacteria were shown to significantly increase lateral root formation and this effect was lost when plants were exposed to indole-deficient bacterial mutants (Bailly et al. 2014). Another common volatile emitted by microorganisms is DMDS (Blom et al. 2011a; Garbeva et al. 2014b; Groenhagen et al. 2014). DMDS is reported to significantly promote plant growth and increase the number of lateral roots and root hairs even at very low concentrations (Meldau et al. 2013). The mechanism of plant growth promotion by DMDS was related to direct increase of sulphur supply. Furthermore, DMDS supplementation significantly reduced the expression of sulphur-assimilation genes as well as methionine biosynthesis and recycling in tobacco plants (Meldau et al. 2013). In contrast to indole and DMDS, some microbial volatiles like hydrogen cyanide and ammonia were determined to be deleterious (Blom et al. 2011a; Kai et al. 2010; Wenke et al. 2010).

The effect of microbial volatiles may be strongly dependent on the ontogenetic stage of the plant. When *A. thaliana* seeds were exposed to volatiles emitted by fungal isolate *Trichoderma atroviride* for 14 days, reduction in plant size, formation of necrotic lesions and loss of chlorophyll was observed (Lee et al. 2015). However, when *A. thaliana* seedlings were exposed to volatiles produced by the same fungus under the same conditions, they exhibited significant increases in growth and chlorophyll production. Similarly, volatile mixtures emitted from the biocontrol fungus *Trichoderma viride* enhanced growth of *A. thaliana* (Hung et al. 2013) and volatiles emitted by *Cladosporium cladosporioides* enhanced growth of tobacco plants (Paul and Park 2013). Overall, the plant's response to growth-promoting volatiles seems to be mediated by auxin, in part due to better iron acquisition and photosynthesis. Furthermore, increased resistance to pathogens can be conferred by exposure to bacterial volatiles, through induction of ISR (induced systemic resistance), and the growth of phytopathogenic fungi can be reduced by exposure to microbial volatiles (see Sect. 8.3.1). Most bacteria activate ISR in plants via a SA-independent pathway involving JA and ethylene signalling. Volatiles produced by *Bacillus amyloliquefaciens* triggered ISR through an ethylene-independent signalling pathway, whereas volatiles produced by *Bacillus subtilis* appear to do this via an ethylene-dependent pathway, albeit independent of the SA or JA signalling pathways (Ryu et al. 2004).

In general, studying volatile-mediated interactions between plants and microorganisms is challenging because of the variation in volatile emission dependent on the physiological state of the producing microorganism and environmental conditions. Additionally, the methods used to study volatile-mediated plant–microbe interactions can lead to different responses in plants and contrasting results, as recently indicated by Lee et al. (2015). Furthermore, plant-associated microorganisms can affect the blend of volatiles released by plants. For example, tomato plants inoculated with the fungal endophyte *Acremonium strictum* emitted diverse terpenes and sesquiterpenes in significantly lower amounts than endophyte-free plants (Jallow et al. 2008). Additionally, endophytic fungi that live within plants can produce many metabolites including volatiles that benefit the host plant. For example *Phoma* spp. isolated from creosote bush produce volatiles that help the shrub to survive harsh desert habitats (Strobel et al. 2011). In a recent study, Peñuelas et al. (2014b) revealed that phyllosphere microbiota can significantly influence plant terpene emissions. Removing floral microbiota of *Sambucus nigra* L. affected both the quality and quantity floral terpene emission (Peñuelas et al. 2014b). Similar studies on the effect of the rhizosphere microbiome as a whole on root volatile production are missing.

8.3.4 Effect of Plant Volatiles on Bacteria

Volatiles produced by plant roots may exert short (μm)- and long (mm)-distance effects on microbes in the rhizosphere. As mentioned above, only a few studies have shown that volatiles produced by roots are also emitted in the rhizosphere (Cecchini et al. 2010; Del Giudice et al. 2008; Kpoviessi et al. 2011; Steeghs et al. 2004; Yeo et al. 2013). Based on in vitro assays, the bioactivities of root-specific volatile terpenoids and phenolic compounds have been associated primarily with growth-inhibiting effects (Wenke et al. 2010). Terpenes and other root-derived VOCs most likely serve multiple roles as C-sources, defence metabolites and chemoattractants. Degradation of plant monoterpenes such as geraniol by soil microbial activity has been demonstrated (Owen et al. 2007), and rhizobacteria such as *Pseudomonas fluorescens* and *Alcaligenes xylosoxidans* have been shown to metabolise α-pinene as their sole carbon source (Kleinheinz et al. 1999). Del Giudice et al. (2008) also reported that bacteria associated with the roots of vetiver grass (*V. zizanioides*) use sesquiterpenes as a carbon source. Many bacterial species use quorum sensing to coordinate gene expression according to the density of their local population. Some plant volatiles may interfere with bacterial quorum sensing (QS) and this can be in both directions. For example, plant volatiles like (+)-enantiomers of carvone, limonene and borneol stimulated bacterial QS, while compounds like α-terpineol and cis-3-nonen-1-ol completely inhibited bacterial QS (Ahmad et al. 2015).

8.3.5 Plant Volatiles in Belowground Plant–Herbivore Interactions

As for aboveground produced volatiles, root volatiles may serve as cues for herbivores to locate their host plant. Belowground herbivores may use CO_2

gradients in the soil to locate roots (Johnson and Nielsen 2012); however, there is some debate on the reliability of such a generic cue. It does not allow herbivores to discriminate between hosts and (toxic) non-hosts, and many other nontarget organisms in the soil produce CO_2 (Erb et al. 2013). Therefore more specific plant volatiles may be better cues for herbivores searching for a suitable host plant. At the same time, these more specific plant volatiles may serve as direct or indirect defences. In particular, volatile products resulting from glucosinolate or cyanogenic glycoside conversion, i.e. cyanides and isothiocyanates, may serve as direct plant defences. They have been found to be toxic or noxious to a wide range of belowground herbivores and pathogens (Hopkins et al. 2009; Kissen et al. 2009; Potter et al. 1998), though specialist herbivores possessing mechanisms to overcome the toxicity of these compounds may use them to locate their host plant. For example, larvae of cabbage white butterflies (*Pieris* spp.) possess specific enzymes to interfere with the formation of ITC which renders the plant less toxic (Wittstock et al. 2003). The adults indeed use ITCs typically produced by cabbages and mustards to locate host plants for oviposition (Hopkins et al. 2009). Similarly, root-feeding herbivores specialised on *Brassica* species, such as the larvae of the cabbage root fly (*Delia* spp.), use ITC to orient towards their food plant in the soil (Fig. 8.1; Kostal 1992). These larvae do not have their own detoxification mechanism but rely on gut microbes to detoxify 2-phenylethyl ITC which is produced upon larval damage (Crespo et al. 2012; Welte et al. 2015).

Plant volatiles are more often studied in their role as indirect plant defences, i.e. to attract natural enemies or predators of herbivores. The evolutionary-ecological framework of indirect defences against arthropod herbivores and the role of plant volatiles therein have been elucidated for aboveground tritrophic interactions since the late 1980s (Dicke and Sabelis 1988; Vet et al. 1991). In one of the first studies showing that indirect defences via volatile emissions function belowground as well, van Tol et al. (2001) reported that entomopathogenic nematodes (EPNs) were attracted to the roots of *Thuja occidentalis* damaged by larvae when given a choice in a Y-tube olfactometer filled with sand. At the time, no specific volatiles were identified. A few years later, it was found in various other plant species that roots damaged by herbivores emit specific mono- and sesquiterpenes (Ali et al. 2010; Rasmann et al. 2005; Steeghs et al. 2004). For example, when damaged by the corn rootworm *Diabrotica virgifera virgifera*, maize roots emit the sesquiterpene (*E*)-β-caryophyllene which attracts EPN that infest and kill the root-feeding larvae (Fig. 8.1; Rasmann et al. 2005). Interestingly, commercial cultivars from the USA have lost the ability to produce this compound, suggesting that the ability to attract natural enemies to the rhizosphere can be selected for (Degenhardt et al. 2009). Restoring the ability to produce (*E*)-β-caryophyllene in one of these varieties, however, also increased its susceptibility to a fungal disease (Fantaye et al. 2015), underscoring the multifaceted function of each volatile compound. Similarly, citrus roots infested by root-feeding herbivores recruit EPN via the emission of several mono- and sesquiterpenes detected in the rhizosphere (Ali et al. 2010). Interestingly, the response of other organisms in the rhizosphere did not always follow this pattern; bacterivorous nematodes that feed on the cadavers of

EPN infested larvae displayed similar behaviours as EPN, whereas nematopathogenic fungi did not seem to respond to these cues (Ali et al. 2013).

Another well-studied plant–herbivore system is the interaction of milkweeds (*Asclepias* spp.) with their specialist root herbivores. Apart from the production of latex containing toxic cardenolides, the roots of these plants also produce various volatiles upon induction by root feeders (Rasmann et al. 2011). These volatiles attract EPN that reduce the impact of the herbivores on plant performance, showing that these rhizosphere volatiles serve as true sensu stricto defences (Karban and Baldwin 1997). In addition, there have been several studies showing that aboveground predators or parasitoids of root herbivores are attracted to infested plants via root-emitted volatiles. For example, ground-dwelling *Aleochara* beetles predating on eggs and larvae of *D. radicum* are attracted by DMDS, a volatile organic compound specifically emitted at high levels by root fly-infested *Brassica* roots (Fig. 8.1; Crespo et al. 2012; Ferry et al. 2007; van Dam et al. 2012). Such volatile cues emitted by herbivore-infested roots could potentially be used by other ground-dwelling or belowground predators such as ants, predatory mites, spiders and even mammals such as moles and rodents (Johnson and Rasmann 2015; van Dam 2009). However, experimental data to support this hypothesis are still lacking.

8.3.6 Plant–Plant Communication

Plants are able to respond to their neighbours in order to avoid competition for light and nutrients. Aboveground, this process is often associated with light perception and involves the emission and perception of ethylene (Kegge et al. 2015; Pierik et al. 2006). In the rhizosphere, root exudates and compounds therein play an important role. One well-studied mechanism for plant–plant communication in the rhizosphere is allelopathy. Allelopathy is a chemical–ecological process in which the secretions or emissions of one plant reduce growth or even kill another plant (Inderjit et al. 2011). Several plant volatiles, including mono- and sesquiterpenes, thiophenes and ITC, have been shown to possess allelopathic properties (Fig. 8.1). In fact, several *Brassica* species are commonly used for biofumigation purposes as the ITCs that are formed upon ploughing reduces weed germination (Vaughn and Boydston 1997). Moreover, it has been shown that sagebrush plants emit various volatiles from the roots that may have an allelopathic effect. MeJA was not among them, even though it has a strong inhibitory effect on the germination of other species (Jassbi et al. 2010). Interestingly, the zone of influence of the allelopathic compounds may be increased by mycorrhizal associations. In an experimental set-up using *Tagetes tenuifolia* plants in a mesocosm, it was shown that common mycorrhizal networks connecting plants may enhance thiophene accumulation away from the rhizosphere of the plant (Barto et al. 2011). In contrast to most studies assessing allelopathic effects of root volatiles, Barto et al. (2011) used a ‘phytometer’ approach to show in vivo that competing plants suffer biomass reductions when growing in soils with higher thiophene accumulations.

Another interesting aspect related to plant–plant communication is the plasticity in root placement. Plants growing next to each other may adapt their root allocation patterns according to their neighbour's identity and even the level of relatedness (Depuydt 2014; Semchenko et al. 2014). It has been experimentally assessed that root exudates can affect the placement of roots away from competitors or kin (Schmid et al. 2013; Semchenko et al. 2014). In recent reviews, most compounds that are listed as being important in such root allocation processes are water soluble and non-volatile (Biedrzycki and Bais 2010; Depuydt 2014). However, based on what is known about the role of volatiles in aboveground plant–plant communication and self-recognition (Heil and Land 2014; Karban et al. 2014a, b), a call for more research on the role of plant volatiles in belowground plant–plant interactions seems reasonable (Biedrzycki and Bais 2010).

8.4 How to Measure Volatiles in the Soil?

Studying the volatiles emitted in the rhizosphere is a challenging task for several reasons. Compared to the aerial headspace of plants, the soil is a dense and heterogeneous matrix, so sampling of rhizosphere volatiles requires more preparation. The first point to consider is the composition of the substrate. For example, the adsorption capacity and smaller grain size of clay will influence the distribution and diffusion of volatile compounds (Barnett and Johnson 2013). In addition, the capacity of the soil to bind water will influence the result of soil volatile trapping experiments, as soil humidity affects the diffusion and distribution of volatiles in the rhizosphere (Hiltpold and Turlings 2008). In a later phase, water in the traps may interfere with chemical analysis by gas chromatography. Those factors may be partially controlled in a greenhouse experiment but not in more realistic field experiments.

Moreover, the properties of the biological system as a whole are of importance for the sampling strategy. In a single species experiment, volatiles emerging from the plant roots and those emerging from the soil can be easily separated by including 'soil blank' samples. When it comes to identifying the volatile profiles of roots growing in a plant community, it gets more difficult. The first question would be how to separate the volatiles of different plant species, especially when the roots are intertwined. Another challenge is to collect plant volatiles in vivo. There are several approaches described in the literature, e.g. dynamic and static headspace sampling of roots in mesocosms, but most of these can only be performed under laboratory conditions. As for every experiment dealing with living organisms, the biggest challenge might be to do the analysis in a non-invasive manner. This is particularly difficult since most of the existing volatile trapping methods rely on inserting sampling devices in the soil next to the root, thereby possibly damaging the root tissues. In the next sections, we discuss different sampling approaches that have been used to sample root or rhizosphere volatiles and evaluate their suitability for root samplings based on published experiments (see also Table 8.1).

Table 8.1 Overview of non-invasive techniques for sampling root volatiles with their benefits and drawbacks as reported in experimental papers (last column)

Sampling technique	Advantages	Disadvantages	References
Solid-phase micro-extraction (SPME)	• Wide range of sorbent materials • Sample enrichment • Easy to use	• Quantification difficult • No resampling • No long-term storage • Fragile fibres	Gfeller et al. (2013), Rasmann et al. (2011), Rasmann et al. (2005), Rasmann and Turlings (2008), Robert et al. (2012), Yang et al. (2013)
Direct thermal desorption (TD)	• Wide range of sorbent materials • Multiple analysis possible (with recollection) • High throughput • Long-term storage	• Tubes costly • Pumps needed in the field (dynamic sampling) • Quantification difficult	Crespo et al. (2012), Harper (2000), Stewart-Jones and Poppy (2006), van Dam et al. (2010)
Conventional traps (GC)	• Wide range of sorbent materials • Multiple analysis possible • High throughput • Long-term storage • Inexpensive • Exact quantification (int. standard)	• Pumps needed in the field (dynamic sampling) • Laborious elution of traps • Prone to contamination during elution	Ali et al. (2010, 2011, 2012)
Proton-transfer-reaction mass spectrometry (PTR-MS)	• Real-time measurement • Dynamic volatile profile	• Expensive equipment • No identification for molecules with same mass • No resampling	Crespo et al. (2012), Danner et al. (2012, 2015), Rostás et al. (2015), Samudrala et al. (2015), Steeghs et al. (2004), van Dam et al. (2012)

Int. internal

8.4.1 *Solid-Phase Micro-Extraction (SPME)*

Solid-phase micro-extraction (SPME) is widely used for the trapping of above-ground plant volatiles (Yang et al. 2013). The advantage of SPME is that the volatiles are enriched on the fibres, which allows the analysis of trace compounds. A large number of fibres with different adsorptive properties are commercially available. In principle, SPME fibres are easy to handle and can be easily inserted in preformed slots in the rhizosphere. SPME is well suited for determining the spatial distribution of soilborne volatiles around a plant, e.g. by sampling at defined depths or distances from the plant. For example, SPME was used to show that (*E*)-β-caryophyllene added to sand diffuses over a distance of 10 cm within half an hour (Rasmann et al. 2005). A drawback is that SPME is more expensive, less useful for high-throughput analysis and less suitable for exactly quantifying volatile emissions (Table 8.1). Using SPME, Rasmann et al. (2011) performed a dynamic headspace sampling of root volatiles of milkweed (*Asclepias syriaca*) released after attack by a root-boring beetle *Tetraopes tetrophthalmus* and studied their attractiveness to EPN (Rasmann et al. 2011). Prior to trapping the volatiles on SPME fibres, plants were removed from the soil and the roots were washed with tap water. The results showed that a mixture of 15 root volatiles was significantly increased after 4 days of root herbivory. In this study, SPME was performed only on ground root material to analyse the total pool of root volatiles and showed that inducibility of volatiles is negatively correlated to the constitutive levels (Rasmann et al. 2011). SPME was also used to analyse root volatiles of maize, cotton and cowpea (Rasmann and Turlings 2008; Robert et al. 2012). In these studies, however, roots were harvested and ground before analysis.

Weissteiner et al. (2012) used SPME to measure volatiles emitted from oak trees infested with cockchafer larvae *Melolontha hippocastani*. In addition to SPME sampling, they also used dynamic headspace with thermal desorption tubes. The root volatiles collected by SPME were later used to estimate the concentration for choice assays (Weissteiner et al. 2012). Gfeller et al. (2013) studied the emission of volatiles from barley roots and their effect on wireworms. In contrast to previous studies, roots were left intact even though they were separated from the shoots. Thus, 29 root volatiles could be identified and the authors were able to show that detection was dependent on the cultivation medium (Gfeller et al. 2013). Taken together, these studies illustrate that SPME can be a powerful tool to sample rhizosphere volatiles, especially for trace analysis. The ease of use also makes SPME suitable for field sampling; however, to our knowledge, no study has been published that applies SPME in field experiments.

8.4.2 Direct Thermal Desorption

In general, direct thermal desorption (TD) is a robust technique to collect plant volatiles. In contrast to SPME, the volatile compounds are adsorbed on trapping material packed in a glass or metal tube. Like the SPME fibres, the adsorbent material can have various compositions depending on the target analytes (Harper 2000). One tube can contain different types of trapping materials (mixed bedding), which increases the range of volatiles that can be trapped (e.g. van Dam et al. 2010). The advantage is that the volatile sample can be analysed as emitted in the field or greenhouse without solvent elution. Another advantage of TD is that samples can be stored over a longer time in capped and cooled tubes. Moreover, recently developed TD instruments allow for sample recollection and enable researchers to perform repeated injections, for instance with different GC columns. Another difference to SPME is that TD tubes have to be used in a dynamic sampling system. Plants are enclosed in glass containers or inert plastic bags to which the trap is attached (Stewart-Jones and Poppy 2006), after which a gas flow is applied by either pushing or pulling air through the tube. A push–pull system is the best option, but this may not be feasible in the field. When working with a dynamic sampling system, the applied flow rates and sampling time are important since they determine the amount of compounds adsorbed. Because TD sampling is often used for assessing environmental air quality, national and international agencies have developed standard methods for sampling procedures. The EPA compendium method TO-17[1] and ISO 16017[2] provide a detailed description of the methodologies used for TD volatile trapping.

Disadvantages of TD are the relatively high cost of the equipment and the trapping tubes, even though they can be reused multiple times. TD tubes were used to identify glucosinolate breakdown products in the headspace of *Brassica nigra* roots infested with cabbage root fly larvae (Crespo et al. 2012). In this study, cooking bags prepared according to Stewart-Jones and Poppy (2006) were used to enclose the root headspace of a potted plant, and TD tubes with mixed Carbopack-Tenax bedding were inserted in the bags (Fig. 8.2). TD tubes can also be used with other sampling materials. In a study on root volatiles of dandelion (*Taraxacum ruderalia*), laboratory silicone tubing (PDMS) was used to collect root volatiles in specially designed mesocosms and inserted in empty TD tubes before desorption (Eilers et al. 2015). Fifteen volatiles could be extracted from the rhizosphere and identified by GC-MS. This is one of the few studies where volatiles from the rhizosphere were trapped in situ. However, this method, like SPME, is a ‘single-shot’ analysis, where volatiles cannot be resampled.

8.4.3 Volatile Trapping with Subsequent Elution

Dynamic headspace sampling can also be combined with conventional solvent-elution traps. Similar to TD, volatiles from the rhizosphere are directed through a glass or metal tube filled with an adsorbent by a push, a pull or a push–pull system. After trapping, the volatiles are eluted from the trap with a defined amount of organic solvent and analysed on a GC. The advantage of this approach is that at this point standard compounds can be added to the solvent, which allows for the normalisation and exact quantification of the data. Another advantage is that the

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[1] EPA compendium TO-17 (version 1999), see http://www.epa.gov/ttnamti1/files/ambient/airtox/to-17r.pdf.

[2] ISO 16017–1:2000, see http://www.iso.org/iso/catalogue_detail.htm?csnumber=29194; for ISO 16017–2:2003, see http://www.iso.org/iso/catalogue_detail.htm?csnumber=29195.

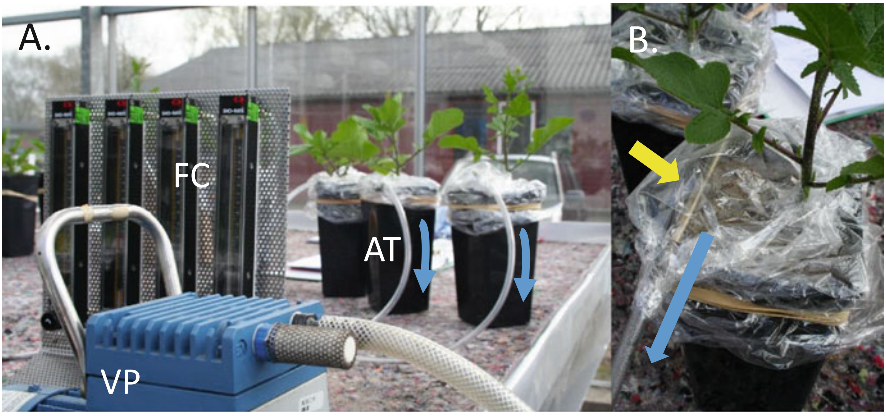

Fig. 8.2 Overview (**a**) and a detail (**b**) of a root headspace sampling set-up using direct thermal desorption tubes (indicated in by the *yellow arrow* in **b**). The *blue arrows* indicate the direction of the airflow. Labels: *VP* vacuum pump, *FC* flow controllers, *AT* air tube. The volume of the root headspace is restricted by mounting a pretreated frying bag around the base of the stem. The tubes are inserted into the bags via slit. Air is pulled over the trap via vacuum pump ('pull' system). For more details, see Crespo et al. (2012). Photographs: Nicole M. van Dam

liquid sample can be stored and injected multiple times. Disadvantages are that it is more labour-intensive, less sensitive due to losses during elution and prone to contaminants and spontaneous conversions in the elution solvent (Table 8.1). Nevertheless, this technique is widely used as no specific TD equipment is needed. For example, solvent-eluted traps were used in a push–pull system to study the emission of citrus root volatiles and their effect on the behaviour of different nematode species (Ali et al. 2011). The authors used a volatile collection apparatus to simultaneously trap below- and aboveground volatiles, allowing a direct comparison of the relationship of both volatile profiles. In a similar study, four major terpenes that were only produced by infested roots were identified (Ali et al. 2010). Finally the same authors conducted a study where they used a soil probe to collect volatiles from infested roots in the field (Ali et al. 2012), a rare example of root volatile trapping outside the laboratory.

8.4.4 Non-invasive Time-Resolved Measurements

All of the above mentioned methods lack temporal resolution, which is an important factor for the understanding of volatile function in an ecological context. SPME, TD and conventional solvent-elution traps mirror only the time interval of the volatile trapping. Dynamic changes in the volatile bouquet within the trapping interval cannot be resolved. Proton-transfer reaction MS (PTR-MS) overcomes this constraint and allows the measurement of plant and root volatiles in real time, which reveals how volatile emissions change over an ecologically relevant timescale (Danner et al. 2012; Steeghs et al. 2004). Besides this great advantage, there are several drawbacks (Table 8.1). After proper calibration and optimisation of the system has been achieved (Samudrala et al. 2015), PTR-MS can be successfully used to analyse particular groups of low molecular weight volatiles. This is illustrated by studies analysing the volatile emissions of *Arabidopsis* or *Brassica* plants both in vitro and in vivo. PTR-MS was successfully applied to follow the volatile emission dynamics of an *A. thaliana* root culture (Steeghs et al. 2004). Interestingly, when these roots were challenged by a root pathogen or a root-feeding aphid, emissions of the monoterpene 1,8-cineole increased. PTR-MS was also used to analyse the root volatiles of weeds, how they are influenced by an endophytic fungus and the response of root herbivores to the changes in root volatiles. In this study, roots were removed from the soil before analysis (Rostás et al. 2015). PTR-MS in situ analyses of volatiles emitted in the root headspace of various *Brassica* species subjected to artificial damage or infested with cabbage root fly larvae revealed that various sulphur-containing compounds show specific dynamic patterns depending on the larval instar of the root herbivore (Crespo et al. 2012) or the *Brassica* species used (van Dam et al. 2012). More recently, using separate cuvettes for sampling roots and shoots, it was shown with PTR-MS that shoot feeders can also significantly enhance DMDS emissions into the root headspace, though not as strongly as local infestation by root-feeding herbivores (Danner et al. 2015). Taken together, these studies illustrate how PTR-MS can acquire time-resolved data on intact plants, even though they were not directly performed in the rhizosphere.

8.5 Discussion

Understanding complex volatile-mediated interactions belowground is a large and intricate puzzle and any attempt to cover this broad topic will remain incomplete. From the evidence in the current scientific literature, it is clear that the two main producers of volatiles belowground are plants and microorganisms. It should, however, be noted that the current lack of knowledge on the emission of volatiles by other rhizosphere organisms, such as nematodes or earthworms, does not mean that they do not produce volatiles that may be relevant for rhizosphere communication. It rather indicates that these groups are currently understudied with regards to this aspect.

Without doubt, plants are involved in intimate interactions with microorganism during their entire life, starting from its infancy as a seed. Investigation of surface-sterilised seeds revealed that the majority of plant species seeds were colonised by bacteria (Cankar et al. 2005; Compant et al. 2005; Graner et al. 2003; Mundt and Hinkle 1976). Molecular methods for detecting seed endophytes (Johnston-Monje and Raizada 2011) revealed distinct community structure between plants as well as between different geographic locations. The zone of influence of the germinating seed has been named the ‘spermosphere’ (Nelson 2004), and the interactions in the spermosphere can be important first steps of the association between bacteria and plant. However, the role of volatiles in spermosphere interactions has not been studied so far.

As indicated in this chapter, several volatiles such as terpenoids and sulphur compounds are commonly produced and emitted by both plant roots and microorganisms. Genomic studies reveal that both groups of organisms carry many genes responsible for the synthesis of such volatiles, possibly with a common evolutionary origin. Based on these commonalities, it is possible that terpenoids and volatile sulphur compounds are a ‘lingua franca’ for inter-kingdom communication between plants, bacteria and fungi.

However, there are several open questions regarding this hypothesis. First, how can an organism distinguish the source of such a common signal? Possibly, this can be achieved by sensing the concentration of the signal similar to what has been reported for quorum sensing. Additionally, the chemical background of other volatiles and non-volatile compounds present in the environment may be important. In aboveground tritrophic interactions, it has indeed been shown that the composition of the background volatile profile is important for the attractiveness of a single volatile to an egg parasitoid (Mumm and Hilker 2005). Finally, for most volatiles, it is as yet unknown exactly how they are perceived by plants. Whereas there is an extensive body of literature on olfactory receptors in insects and mammals, molecular receptors for the perception of terpenoids, for example, have not yet been identified in plants, even though the roles of volatiles in plant–plant interactions were one of the first to be recognised (Heil 2014). The current view is that due to their lipophilic nature, volatiles such as mono- and sesquiterpenes may interfere with membrane structures, thereby causing depolarization of the membranes and triggering Ca^{2+} signalling in plants (Heil 2014; Chap. 12). However, this constitutes a very unspecific mechanism, which raises the question whether such volatiles per se may serve as reliable infochemicals at all (Dicke and Sabelis 1988). It is thus not surprising that the search for volatile receptors in plants was recently coined as one of the ‘hot topics’ in the field (Heil 2014). For microbes, it may be easier to elucidate how volatiles are perceived, as they are more easily transformed and screened for mutations in a high-throughput manner. This facilitates the generation of transformants overexpressing certain volatile production genes, mutants lacking a response to certain volatiles or the use of genetic markers, such as green fluorescent protein (GFP), which may reveal genes that are activated during volatile communication. In such experiments, it will be of utmost importance to mimic common natural conditions, especially with regards to the nutrient level. It was found that the emission of a certain volatile is conditional and may not occur under the nutrient-rich conditions (Garbeva et al. 2014a, b).

A further major challenge is to correctly identify the origin of any particular volatile belowground, especially since many volatile compounds are produced only as a result of interactions. For plant–insect interactions it, has been known for decades that specific volatiles are only produced by a plant when attacked by an herbivore (Heil 2014). The same seems to be true for soil-dwelling bacteria and fungi that respond to each other’s presence by producing (antibiotic) volatiles

(Garbeva et al. 2014b; Kai et al. 2007). It is one thing to sample and detect such compounds when both interaction partners are growing as isolates in a Petri dish on different sides of a divider, but it will be quite another to identify individual compounds in a fully populated rhizosphere where the organisms of interest may not be the most abundant and many other organisms may interfere with the communication. The latter may apply when other bacterial species in the rhizosphere consume or convert the volatile signal before it has reached the receiver. The same may be true for plant-emitted compounds. Thus, when sampling living soils for volatiles, the volatile profile that is found is mostly a mix of originally emitted compounds and catabolic products thereof.

One approach to distinguish the originals might be to first extract roots destructively or to measure emissions from a sterile plant. However, the medium in which the plants are grown strongly affects the volatile profiles (Jassbi et al. 2010). In that sense, labelling organisms with stable isotopes may be a better approach to follow the fate of volatiles in the rhizosphere. Moreover, the conversion of the original signal by a third-party organism does not necessarily lead to distorted communication, as it may provide additional information on potential competitors. Such studies should preferably be conducted in soil mesocosms where the number of interactors, the conditions and the substrate can be somewhat controlled. It should also be considered that prefabricated slits or tubes for inserting sampling devices into the soil mesocosms would be ideal to prevent root damage.

Currently there is an increased interest in using PGPR and other beneficial microbes such as *Trichoderma* and mycorrhizal isolates for sustainable agriculture (Mendes et al. 2013; Raaijmakers et al. 2009). However, experimental additions of beneficial microbes to existing soil communities often fail. Apparently, it is difficult for the microbes that are added to establish in the standing soil communities, which prevents farmers from reaping the full benefits. A greater understanding of the role of volatile communication in rhizosphere processes may help to increase the efficacy of such novel approaches. All in all, it is due time to open the black box of the soil a bit further and stick our noses in it to 'sniff out' the compounds that mediate the many interactions belowground. This may not only lead to a better understanding of the role of volatiles for belowground communication but also increase the potential to find sustainable solutions for agriculture and novel agrochemicals.

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Chapter 9
Complexity of Plant Volatile-Mediated Interactions Beyond the Third Trophic Level

Erik H. Poelman and Martine Kos

Abstract Food chains of plant-associated communities typically reach beyond three trophic levels. The predators and parasitoids in the third trophic level are under attack by top predators or parasitised by hyperparasitoids. These higher trophic level organisms respond to plant volatiles in search of their prey or host. Thereby, plant volatiles affect community processes such as competition and intraguild predation among predators and parasitoids at the terminal end of the food chain. The response of fourth trophic level organisms to plant volatiles potentially reduces the benefit of these volatiles as indirect defence for the plant. In the application of parasitoids as biological control agents of herbivore pests, hyperparasitoids may diminish the effectiveness of parasitoids. Detailed understanding of the use of plant odours by hyperparasitoids may provide tools to further optimise biological control.

9.1 Introduction

Plant volatiles mediate many of the most critical processes that determine plant performance and fitness. Plants use volatile compounds as a fast track for internal communication between organs that are physically nearby but more distant through the sap stream, as found for leaves on two branches of a tree. Moreover, plants

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perceive volatile information from neighbouring plants, which allows them to adjust their growth or defence strategy depending on the information perceived (see Chap. 7). Volatiles also function in direct plant resistance to herbivory by repelling specific herbivores, with a downside of also becoming apparent to others (see Chaps. 5 and 6). In animal-pollinated plants, floral scents primarily drive reproduction by attracting vertebrate or insect pollinators (see Chaps. 10 and 11). The specific volatiles that are induced by herbivore feeding are used by predators and parasitoids of herbivores to locate their prey or host (see Chaps. 5 and 6). These herbivore-induced plant volatiles (HIPVs) thereby provide plants with indirect resistance through top-down control of their herbivore attackers. Ever since the seminal paper by Price and colleagues (1980), it has become evident that such responses of the third trophic level parasitoids and predators strongly determine community processes and affect evolution of both plant and herbivore traits.

HIPVs have been hypothesised to mediate indirect defence in which plants attract enemies of their own herbivore enemies and by doing so reduce the impact of herbivory on plant fitness. Although several laboratory and field studies have found that, in isolation of other community members, parasitism and predation of herbivores may indeed reduce fitness costs of herbivory (van Loon et al. 2000; Smallegange et al. 2008; Schuman et al. 2012; Gols et al. 2015), these effects have not been evaluated in a full community context. Release of volatiles in a community context and the multifunctionality of volatile communication outlined in this book means that this source of information may potentially be used by other organisms that may impact plant fitness (Kessler and Heil 2011). Although in virtually all plant species HIPVs do attract parasitoids and predators, it is now well established that plants that emit HIPVs may attract additional herbivore species and may have reduced pollinator visitations, each reducing the plant fitness benefit of volatile release (Kessler and Heil 2011). Moreover, parasitoids and predators are not at the end of the food chain and are therefore not necessarily the ultimate receptors or beneficiaries of HIPVs. Organisms at the fourth trophic level may also use plant volatiles to find their host or prey, potentially diminishing the benefit of attracting third trophic level parasitoids and predators (Poelman et al. 2012).

In this chapter, we review the use of HIPVs by organisms at the trophic end of a food chain, such as vertebrate predators and fourth or higher trophic level insects such as hyperparasitoids and predators or parasitoids involved in intraguild predation (Fig. 9.1). We discuss how response to HIPVs by organisms at higher trophic levels affects community processes and what role these processes play in applications such as in biological control. We provide future directions for the studies of higher trophic level organisms and their responses to HIPVs and identify opportunities for the use of HIPVs in reducing the impact of hyperparasitoids on biological control.

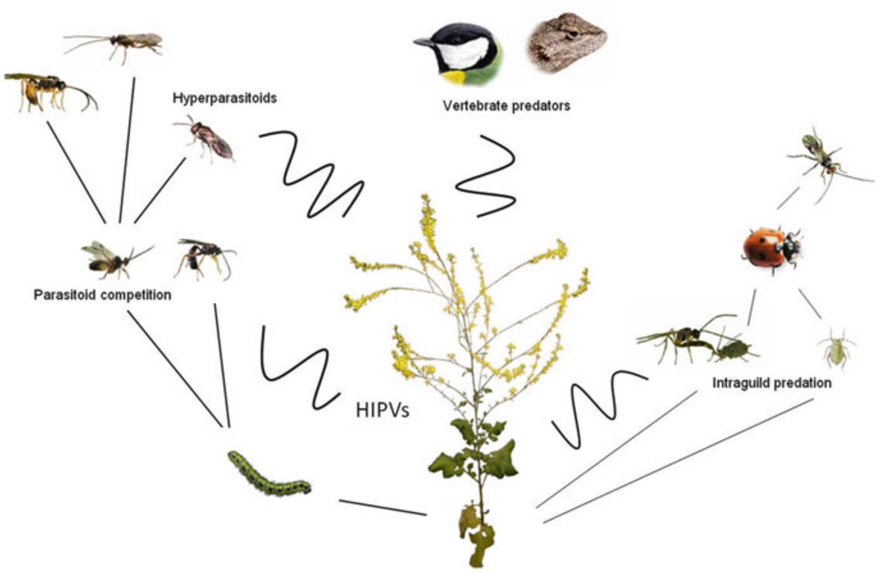

Fig. 9.1 Herbivore-induced plant volatiles (HIPVs) attract third trophic level parasitoids, invertebrate and vertebrate predators. Moreover, predators and hyperparasitoids at the terminal end of the food chain in the fourth or higher trophic levels make use of HIPVs in host or prey location as well. HIPVs thereby play important roles in community processes such as competition among parasitoids and predators for hosts or prey as well as intraguild predation among higher trophic level organisms

9.2 Vertebrate Predators Responding to HIPVs

Although the role of HIPVs in attraction of insect predators or parasitoids is well established, the role of HIPVs in attracting vertebrate predators, which are often voracious consumers at the terminal end of the food chain, has received little attention. Nevertheless, willow warblers (*Phylloscopus trochilus*), a passerine bird species, were found to discriminate between branches of mountain birch trees that were infested with sawfly larvae and branches of undamaged trees. These interactions were potentially odour mediated but could have been mediated by vision as the UV spectra of induced leaves are altered by increased flavonoid concentration (Mäntylä et al. 2004). By placing induced trees in screened compartments in a choice test between herbivore-damaged and herbivore-undamaged trees, Amo et al. (2013) confirmed that great tits (*Parus major*) use HIPVs in determining the location of their herbivorous prey. Great tits more frequently flew to herbivore-induced trees over undamaged trees, even when the screens were of such fine mesh that birds could not visually perceive the tree and had to rely on tree odours that passed through the screen. By correlating herbivore-induced tree odour blends with bird predation rates on insect clay models representing artificial moth larvae in the field, Mäntylä et al. (2014) found that α-pinene concentrations may be used by birds to locate herbivore-rich trees. Lizards (*Aspidoscelis tigris*, *Uta stansburiana* and *Sceloporus magister*) also use olfaction in location of their caterpillar prey and benefit from plant-derived compounds. When large tobacco hornworm (*Manduca sexta*) caterpillars feed on *Nicotiana* plants, they ingest the plant-defensive trichomes that contain toxic acyl sugars. The hornworm is resistant to these sugars, and the digested acyl sugars break down into branched chain aliphatic acids that add a distinct smell to the caterpillar frass. The lizards use these plant-derived compounds in the frass to locate plants under attack by caterpillars (Stork et al. 2011). These studies indicate that vertebrate predators can use HIPVs in an analogous fashion to insect predators and parasitoids.

9.3 Hyperparasitoids Responding to HIPVs

Hyperparasitoids, i.e. secondary parasitoids that develop inside or on the body of primary parasitoids and ultimately kill their host, commonly occupy the fourth trophic level in plant–insect food chains (Sullivan and Volkl 1999). Thus far, only limited information on the foraging behaviour of hyperparasitoids and the cues involved in host location is available (Sullivan and Volkl 1999; Poelman et al. 2012). Based on the host developmental stage that they attack, hyperparasitoids are generally classified into two groups: primary and secondary hyperparasitoids. Primary hyperparasitoids (or endohyperparasitoids) are mostly koinobionts that oviposit in the larvae of their hosts and allow the host to continue feeding and growing during parasitism, whereas secondary hyperparasitoids (or ectohyperparasitoids) are primarily idiobionts that feed externally on nongrowing host stages such as eggs and pupae or hosts that have been paralysed before oviposition (Askew and Shaw 1986; Sullivan and Volkl 1999; Harvey et al. 2012).

Endohyperparasitoids are usually more host specific than ectohyperparasitoids, because they need more physiological adaptations to survive, such as a mechanism to overcome the immune system of the host (Sullivan and Volkl 1999). Because of their narrow host range, endohyperparasitoids have been hypothesised to use volatiles from their host plant for host location (Sullivan and Volkl 1999). However, studies on effects of plant cues on endohyperparasitoid foraging behaviour have shown contrasting results. For example, Singh and Srivastava (1987a, b) showed that the aphid endohyperparasitoid *Alloxysta pleuralis* is attracted to foliage extracts of host plants. In contrast, Buitenhuis et al. (2005) reported no attraction of two species of aphid endohyperparasitoids to plant odours. Because they often have a broad range of hosts on a broad variety of host plants, ectohyperparasitoids were previously assumed not to rely on plant volatiles for host location (Sullivan and Volkl 1999; Volkl and Sullivan 2000; Buitenhuis et al. 2005). Instead, ectohyperparasitoids were expected to mainly use cues derived directly from the parasitoid or herbivore host, such as cues arising from the silky parasitoid cocoon or aphid honeydew (Sullivan and Volkl 1999; Volkl and Sullivan 2000; Buitenhuis et al. 2004). Some ectohyperparasitoids, such as the aphid hyperparasitoid *Dendrocerus carpenteri*, were shown not to respond to any cues from hosts or host plants and to search for a host plant at random (Sullivan and Volkl 1999). In contrast, some studies have reported that specialised ectohyperparasitoids can use plant odours for host location. For example, the specialised ectohyperparasitoid *Euneura augarus*, which only attacks aphids on conifers, preferred volatiles from conifers over volatiles from non-coniferous plants, both in the absence and presence of aphid mummies (Volkl and Sullivan 2000). Although several of these studies show that plant volatiles play a role in host habitat location by hyperparasitoids, relatively little is known about the use of more specific host-related cues such as HIPVs or host infochemicals that allow hyperparasitoids to more reliably determine host presence.

Recent findings for a single herbivore–parasitoid–hyperparasitoid food chain show that hyperparasitoids may also use HIPVs and herbivore-derived cues for host location (Poelman et al. 2012; Zhu et al. 2014, 2015). In behavioural experiments, the secondary (ectophagous) hyperparasitoid *Lysibia nana*, which only attacks pupae of parasitoids in the genus *Cotesia*, preferred odours from herbivore-infested plants over odours from uninfested plants (Poelman et al. 2012; Zhu et al. 2015). Furthermore, plants induced by parasitised herbivores emitted different volatile blends than plants induced by unparasitised (healthy) herbivores, and *L. nana* was able to differentiate between these volatile blends and preferred volatiles from plants infested with parasitised herbivores (Fatouros et al. 2005; Poelman et al. 2012; Zhu et al. 2015). Even when the host larvae were developing in different herbivore species, the hyperparasitoids were able to use HIPVs to find their host (Zhu et al. 2015). This suggests that third trophic level parasitoids have a strong effect on their herbivore host during development and that this results in HIPV signatures that reveal their presence to their own hyperparasitoids enemies. While developing in their host, the parasitoid larvae alter the host's physiology for their own benefit. These physiological changes result in changes in the composition of herbivore oral secretions that contain elicitors crucial for plant responses to herbivory, including HIPV responses. Because third trophic level parasitoids differ in their host manipulation, their extended phenotypes of HIPV induction through their herbivore host also differ (Poelman et al. 2012), and this is again largely driven by how each parasitoid species specifically affects herbivore oral secretions (Poelman et al. 2011). As a consequence, this may even allow hyperparasitoids to discriminate between plants under attack by herbivores in which different species of parasitoids develop and may provide cues of host quality or suitability to hyperparasitoids (Poelman et al. 2012). Hyperparasitoids also fine-tune their host location when arriving on the plant by using infochemicals derived from the parasitised caterpillar. The hyperparasitoid *Baryscapus galactopus* uses herbivore body odours to locate parasitised herbivores, which differ in odour emission from unparasitised herbivores (Zhu et al. 2014). These findings demonstrate that hyperparasitoids can use HIPVs and herbivore infochemicals to locate their host.

9.4 Intraguild Predation

Plant volatiles may also mediate interactions among third trophic level organisms and thereby affect competition among those organisms or mediate the frequency of intraguild predation. Intraguild predation occurs when two species compete for the same host or prey and one (or both) of them also feeds on the competitor (Rosenheim et al. 1995; Müller and Brodeur 2002; Vance-Chalcraft et al. 2007). Intraguild predation may be asymmetrical, when species A feeds on species B but not vice versa, or symmetrical, when both species feed on each other (Rosenheim et al. 1995; Müller and Brodeur 2002). If one (or both) of the carnivore species is attracted to HIPVs, the emission of these volatiles may enhance the likelihood of intraguild predation, potentially resulting in a reduction in the top-down control of herbivores. Here, we discuss several forms of intraguild predation between predators and parasitoids and the role of HIPVs in these interactions.

9.4.1 Parasitoids of Predators

Parasitic insects have diversified to exploit the many different types of hosts that are found in the arthropod phylum. This includes hymenopteran and dipteran parasitoids that lay their eggs in or on predatory insects, a common case of intraguild predation in insect communities (Kohler et al. 2008). As predators and their infochemicals are generally inconspicuous, parasitoids may rely on cues associated with predator habitat when locating their predatory host. Herbivore-induced plant volatiles may provide these parasitoids with a detectable cue that predicts the presence of predators. Parasitoids of hoverfly larvae that feed on aphid colonies have been found to use volatiles from aphid-infested plants to locate habitats that may contain hoverfly larvae (Rotheray 1981). After arrival on such plants, the parasitoids may use infochemicals derived from the hoverfly larvae directly or may potentially make use of aphid alarm pheromones released by aphids under attack by predators. The use of indirect predictors in host location by parasitoids of predators has received little attention to date, but it is likely that herbivore-induced plant odours play a role for many of these species. This is supported by studies that monitored responses of insects to synthetic plant volatiles. In a cabbage field, traps baited with the plant volatile methyl salicylate not only attracted primary parasitoids but also the parasitoid *Anacharis zealandica* that lays its eggs in the predaceous lacewing larvae (Orre et al. 2010).

9.4.2 Parasitoid Competition

A single herbivore species is often a host for several parasitoid species that in turn are under attack by a community of hyperparasitoids. In parasitoid communities, there is frequently competition for hosts (Harvey et al. 2013). Competition may occur intrinsically when two parasitoids parasitise the same herbivore host, and their larvae compete for the limited host resources. The adult parasitoids compete extrinsically for the limited number of hosts available. In the latter process, volatiles play a major role. First, plant species, cultivar and genotypes differ in their response to herbivory including the quality and quantity of HIPVs emitted under herbivore attack. Plants that strongly respond to herbivore feeding by producing HIPVs attract more parasitoids of different species than plants that have less attractive HIPVs (Poelman et al. 2013). Plant variation in volatile emission thereby generates a heterogeneous landscape for the intensity of parasitoid competition. Second, parasitoid species differ in their host location strategies, which are often associated with how parasitoids exploit volatile information. Parasitoids with a narrow host range may use more specific volatile cues to locate their host than parasitoids with a wider host range. Moreover, parasitoids that search for gregarious hosts that feed in aggregations and have a clumped distribution may more strongly associate specific volatiles with their reward to fine-tune host location strategies. Parasitoids that search for solitary hosts that are typically found in ideal free distributions may benefit less from optimising their host location by volatile-associated learning (Vos and Hemerik 2003). These different strategies of volatile use may determine parasitoid competitiveness in different habitats. Third, parasitoids can directly use plant volatile information to avoid competition; *Cotesia glomerata* has been found to avoid plants that were previously visited by other parasitoids based on variation in HIPVs induced by parasitised and unparasitised hosts (Fatouros et al. 2005).

9.4.3 Predators Eating Parasitised Herbivores

When predators feed on herbivores that have already been parasitised, it results in asymmetrical intraguild predation (Rosenheim et al. 1995; Müller and Brodeur 2002). This form of intraguild predation is, for instance, prevalent in aphid communities, where predators such as ladybird beetles and lacewings often consume parasitised aphids and aphid mummies (aphid remains containing a parasitoid pupa) (Brodeur and Rosenheim 2000; Colfer and Rosenheim 2001; Meyhofer and Klug 2002). Although most studies on effects of HIPVs on carnivore behaviour have focussed on parasitoids, predators have also been found to use HIPVs to locate their prey. This has been shown mostly for predator taxa that feed on aphids, such as hoverflies, ladybird beetles and lacewings (Steidle and van Loon 2002; Zhu and Park 2005; Harmel et al. 2007; Verheggen et al. 2008). As aphid parasitoids are also attracted to aphid-induced plant volatiles (Bradburne and Mithen 2000; Blande et al. 2007; Kos et al. 2013), the emission of these HIPVs may increase the likelihood of intraguild predation by aphid predators on aphid parasitoids. On the other hand, some predators may avoid ovipositing on plants containing parasitised herbivores. For instance, females of the hoverfly *Episyrphus balteatus* did not oviposit on plants containing mummified aphid colonies (Pineda et al. 2007), probably because their offspring lack the capability to open the mummy shelter with their mouthparts and hence cannot feed on this resource (Meyhofer and Klug 2002; Pineda et al. 2007). As discussed above, plants induced by parasitised herbivores may emit different volatile blends than plants induced by healthy herbivores, and (hyper)parasitoid wasps can discriminate between these volatiles (Fatouros et al. 2005; Poelman et al. 2012; Zhu et al. 2015). It is possible that predators may also use such differences in HIPVs to discriminate between plants infested with parasitised and healthy herbivores, but this remains to be studied. It may be expected that especially predator species whose larvae are physically unable to feed on parasitised herbivores or are unable to recognise parasitised herbivores as valuable prey may have evolved to use HIPVs to avoid plants containing parasitised herbivores, but future studies should determine whether this is true.

9.5 HIPVs, Community Organisation and Plant Fitness

As plant volatiles are used by numerous organisms in the second and higher trophic levels, plant volatiles are an important plant trait that affects community organisation. The variation of volatile blends across plant species, genotypes and the specificity of odour blends induced by different herbivores causes a heterogeneous odour landscape. Such heterogeneity contributes to determining the species composition of plant-associated communities but also the strength of interactions among the species that are part of that assembly (Poelman et al. 2013). As outlined in the previous paragraphs, HIPVs strengthen the consumptive interaction between predator and prey but at the same time may also increase competition among higher trophic level organisms, as well as promote secondary or intraguild predation. These processes may strongly determine top-down control of herbivores and the pressure that the herbivores exert on plants and thus the fitness benefit or defensive nature of plant volatile release. Many of these processes indeed determine abundance of herbivores and predators by population dynamic processes. However, whether and how strongly these processes contribute to plant fitness associated with volatile release remains to be identified (Kessler and Heil 2011; Gols 2014; Poelman 2015). The use of HIPVs by hyperparasitoids, for example, results in population control of primary parasitoids that release plants from herbivore pressure, which may have fitness consequences for the plant. The event of hyperparasitism, however, does not directly interfere with the current fitness outcome of the interaction between plant, herbivore and parasitoid. Hyperparasitism does not cure the herbivore from its parasitoid: the herbivore will die at a similar age when the parasitoid larvae egress from its body and pupate, after which the hyperparasitoid larvae will kill their parasitoid host. From an individual plant's perspective, attraction of parasitoids by HIPVs releases the plant from its current attacker regardless of subsequent attraction of hyperparasitoids. This may, however, come with costs of reduced abundance of beneficial parasitoids when a second generation of herbivores arrives (Poelman et al. 2012).

9.6 Biological Control

Biological control, in which natural enemies such as parasitoids and predators are used to reduce pest populations, is considered the most environmentally safe and cost-effective pest management method that is currently available (van Lenteren 2012). However, biological control agents are themselves parasitised or preyed upon by organisms at the fourth trophic level. Predation or parasitism on species that are introduced as biological control agents represents a major constraint for the efficacy of biological control and is often used to explain the failure of biological control programmes (Stiling 1993; Schooler et al. 2011). In this section, we first describe the challenges that biological control of pests faces due to hyperparasitism and intraguild predation of the biological control agents, especially when using synthetic HIPVs to attract carnivorous insects. We then give an example of how plant volatiles can be exploited to manipulate trophic interaction webs to improve the efficacy of biological control.

9.6.1 Hyperparasitism in Biological Control

Hyperparasitoids develop inside or on the body of primary parasitoids, the most commonly used agents in classical biological control of insect pests. Several studies showed that the presence of hyperparasitoids can reduce the efficacy of biological control (reviewed in, e.g. Rosenheim et al. 1995; Sullivan and Volkl 1999). Hyperparasitoids can affect primary parasitoid populations not only directly, by killing the parasitoid larvae, but also indirectly, by inducing patch-leaving behaviour in the adult parasitoids (Holler et al. 1994; Petersen et al. 2000). Disruptive effects of hyperparasitoids on biological control have been mostly studied in aphid–parasitoid–hyperparasitoid communities (Sullivan and Volkl 1999). Several studies showed that the level of hyperparasitism of aphid parasitoids in the field can be very high, reaching up to 100 % towards the end of the growing season (e.g. Holler et al. 1993; Gomez-Marco et al. 2015). An experimental study demonstrated that hyperparasitoids can completely eliminate the parasitoid *Aphidius ervi*, a biological control agent of the pea aphid *Acyrthosiphon pisum*, under controlled conditions in the greenhouse (Schooler et al. 2011). However, other studies found little or no proof that hyperparasitoids can disrupt biological control by parasitoids. This was attributed to their relatively low lifetime fecundity, a lack of synchrony between the primary and hyperparasitoid or the finding that in diverse communities in the field other parasitoids that are less vulnerable to hyperparasitoids can take over the function of vulnerable ones (Mackauer and Volkl 1993; Sullivan and Volkl 1999; Acebes and Messing 2013; Nofemela 2013). Some studies suggested that hyperparasitoids can even have a beneficial effect on biological control, because they can dampen extreme host–parasitoid oscillations (Beddington and Hammond 1977; Sullivan 1987).

Exploiting plant volatiles to manipulate the behaviour of natural enemies, e.g. in push–pull strategies, is a commonly proposed strategy for improved biological control of pests (Cook et al. 2007). For instance, aphid-induced plant volatiles have been shown to be attractive to aphid parasitoids and can be used to lure these parasitoids into the crop field (Powell and Pickett 2003). Many other studies have demonstrated that the application of synthetic HIPVs in lures or traps can increase the recruitment and retention of parasitoid wasps (e.g. James and Price 2004; James 2005; James and Grasswitz 2005; Kaplan 2012). However, as discussed in Sect. 8.3, hyperparasitoid wasps may also use HIPVs for host location (Poelman et al. 2012; Zhu et al. 2015). Hence, there is a risk involved in applying synthetic HIPVs for enhanced attraction of parasitoid wasps, as this may, unintentionally, enhance attraction of hyperparasitoid wasps. The same risk applies to the use of breeding to produce cultivars that emit volatile compounds that are particularly attractive to biological control agents, another method that has been suggested for enhanced crop protection (Bottrell et al. 1998; Gouinguene et al. 2001; Hoballah et al. 2002; Kaplan 2012). Previous work with several cultivars of *Brassica oleracea* showed that cultivars that were more attractive to primary parasitoids were also more attractive to their hyperparasitoids (Poelman et al. 2013). To ensure a realistic assessment of whether applying synthetic HIPVs or breeding for attractive cultivars increases the efficacy of biological control, it is important that future studies take effects on hyperparasitoids into account.

9.6.2 Intraguild Predation in Biological Control

For biological control of arthropod pests, several forms of intraguild predation can pose a problem, such as predators that attack each other, predators that attack herbivores containing a developing parasitoid and facultative hyperparasitoids that can behave as primary as well as secondary parasitoids (i.e. that can parasitise both herbivores and primary parasitoids) (Rosenheim et al. 1995; Brodeur and Rosenheim 2000; Müller and Brodeur 2002). Intraguild predation, primarily when predators are involved, has been demonstrated to be disruptive for biological control in several cases (reviewed in Rosenheim et al. 1995). For example, biological control of spider mites in California cotton often fails, even though there are at least four predator species present that, when tested singly, can effectively suppress this pest. This failure of biological control by the full predator community is likely caused by intraguild predation (Rosenheim 2005). In other cases, intraguild predation did not disrupt biological control. For example, despite the negative impact of the ladybird beetle *Hippodamia convergens* on populations of the aphid parasitoid *Lysiphlebus testaceipes* in cotton, population suppression of cotton aphids was greatest when both ladybird beetles and parasitoids were present (Colfer and Rosenheim 2001).

As discussed above for hyperparasitism, the use of synthetic HIPVs in biological control may also increase the likelihood of intraguild predation. For example, the study by James (2005) tested effects of 15 HIPVs on attraction of different natural enemies. For several of the tested compounds, multiple species or families of natural enemies, including predators and parasitoids, were simultaneously attracted to sticky traps baited with single synthetic HIPVs (James 2005). A meta-analysis by Rodriguez-Saona et al. (2011) also showed attraction of different carnivore taxa, including parasitoids and generalist predators like ladybird beetles, lacewings and hoverflies, to a single synthetic HIPV, methyl salicylate. Another study showed that traps with methyl salicylate not only attracted more primary parasitoids but also more parasitoids of lacewings (Orre et al. 2010). Together, these studies suggest that the risk of intraguild predation may increase when using synthetic HIPVs to enhance attraction of natural enemies to crops, which may result in a reduction in the top-down control of pests. Therefore, future studies on using synthetic plant volatiles with the aim of improving biological control should take potential effects on intraguild predation into account.

9.6.3 Exploiting Plant Volatiles to Reduce Hyperparasitism in Biological Control

Push–pull strategies are commonly proposed strategies for attracting biological control agents to crops (Cook et al. 2007) but may also be used to control hyperparasitoids, for instance, by assembling attractive lures based on plant volatiles that can be used in traps to divert hyperparasitoids away from the biological control agents. To use this strategy for hyperparasitoids, a thorough understanding of the behavioural–chemical ecology of hyperparasitoids is required. Unfortunately, very limited information on the foraging behaviour of hyperparasitoids is available, although new findings show that hyperparasitoids can use plant volatiles to find their host (Poelman et al. 2012; Zhu et al. 2015; see also Sect. 8.3). Future studies should identify which volatile phytochemicals are attractive to different hyperparasitoid species and which concentrations and combinations of compounds are most effective in luring the hyperparasitoids away from the crop. It is important that these lures do not interfere with the activity of the biological control agent itself. Primary parasitoids often rely on highly specific information from both the plant and the herbivore species for host location and host selection (Vet and Dicke

1992; Dicke 1999; Takabayashi et al. 2006). In contrast, hyperparasitoids often have a broad host range, with their hosts occurring on a broad variety of host plants, and hence may depend less on specific information (Sullivan and Volkl 1999). This may suggest that lures composed of general plant cues have the highest potential in luring away the hyperparasitoids, without affecting the behaviour of the primary parasitoids, but future studies should determine whether this is true. Moreover, it is known that hyperparasitoids can distinguish between volatile blends from plants induced by parasitised and unparasitised herbivores (Poelman et al. 2012; Zhu et al. 2015) and that primary parasitoids themselves can use this information to prevent competition (Fatouros et al. 2005). Hence, there may be potential in finding volatiles that are attractive to hyperparasitoids but unattractive or even repellent to primary parasitoids, in the headspace of plants infested with parasitised herbivores.

9.7 Future Directions

The few studies on vertebrate predators and hyperparasitoids currently conducted highlight that these organisms respond to small changes in plant volatile headspaces to locate their prey or host, and volatiles thus affect organisms at the terminal end of the food chain. Because these organisms have been under-sampled, little is known about general rules in how hyperparasitoids associated with different herbivore–parasitoid food chains, e.g. aphid versus caterpillar and generalist versus specialist associations, can rely on volatile information in host location. This includes the mechanisms underlying volatile emission induced by parasitised herbivores that has been identified for only a single caterpillar study system and is unknown for aphid-associated parasitoids. The notion that volatile release can also affect community processes such as competition among parasitoids or intraguild predation calls for studies that evaluate how variation in volatiles affects co-existence of species-rich parasitoid communities associated with a single host. Identification of the compounds to which hyperparasitoids respond may provide the potential to manipulate host–parasitoid–hyperparasitoid interactions and dampen the negative effect of hyperparasitoids on parasitoids used in biological control.

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Chapter 10
On the Air: Broadcasting and Reception of Volatile Messages in Brood-Site Pollination Mutualisms

Renee M. Borges

Abstract Brood-site pollination mutualisms are those in which plants offer sites to pollinators for the development of offspring or mimic the presence of these sites in exchange for pollination services. Floral scent is an important component of pollinator attraction in such mutualisms and is often composed of volatiles that are commonly emitted by plants in other contexts. Therefore, private channels that employ unusual scent compounds are not the norm. Pollinators must make sense of the volatile messages broadcast by plants against the ensuing background volatile noise using a combination of strategies at the peripheral olfactory system and at higher processing levels. Pollinator reproduction, parts of which occur on or within the host plant, imposes special restrictions on partner compatibility within brood-site pollination systems. A comprehensive understanding of constraints on volatile broadcasting and reception within ecological and evolutionary contexts in this cross-kingdom communication must inform and guide future research in this area.

> I think the king is but a man, as I am: the violet smells to him as it doth to me: the element shows to him as it doth to me.
>
> Shakespeare, *Henry V* (4.1.155-7)

> It is a recognized fact that smell, ordinary smell, the smell that affects our nostrils, consists of molecules emanating from the scented body..... But what is materially emitted by the female Bombyx or Great Peacock? Nothing, according to our sense of smell. Should science one day, instructed by the insect, endow us with a radiograph of smells, this artificial nose will open out to us a world of marvels.
>
> Jean-Henri Fabre, *The Life of the Caterpillar*, 1916

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10.1 Introduction

Shakespeare, the intelligent sixteenth century layman, was inspired to write of the similarity in olfactory perception within the human species. Four centuries later, the accomplished naturalist Jean-Henri Fabre hoped that science would solve the mystery of interspecies differences in scent perception. A century after Fabre, this chapter reviews what science has revealed about cross-kingdom communication using plant volatiles.

10.1.1 Scent Broadcasting by Plants for Pollination: Ecological Contexts

The immobility of plants places a special constraint on how they exchange gametes with each other. Excluding plants that use wind as a gamete motility agent, individual plants can move gametes within themselves or between other plants by engaging with the sensory systems of mobile animals and attracting them to visit reproductive structures either in an honest exchange of rewards or by deceiving them into visiting these structures even when no rewards exist. Plants may engage with the sensory modalities of vision, olfaction, gustation and thermal perception of their pollinators and offer rewards such as adult and larval food, mating sites, brood sites for offspring development, refuges from inclement weather and constituents of mating pheromones. While visual, thermal and gustatory signals are important and act at different scales, this chapter will focus only on olfactory signals. Furthermore, of all the possible rewards, this chapter will concentrate on brood sites as pollination benefits and on systems where plants may deceive pollinators about the presence of brood sites when none actually exist.

Brood-site or nursery pollination mutualisms are special cases of pollination mutualisms in which pollinators oviposit and their offspring develop within the plants that they pollinate (Sakai 2002). There are three major classes of these mutualisms: those where the brood are ovule or seed parasites, those where brood largely feed on pollen of fresh flowers and those whose brood feed on decaying tissues of flowers after pollination; the last category usually comprises the male parts of flowers in hermaphroditic species or staminate flowers in dioecious species (Sakai 2002; Dufaÿ and Anstett 2003). Another new class of brood site involves nesting space rewards to pollinators within domatia of ant-plants (Shenoy and Borges 2008).

A successful plant–pollinator relationship will exist when constraints are strategically overcome, and a net win–win outcome has been achieved. For a communication system between plants and pollinators that is based on olfaction, this will require that (1) volatiles associated with floral structures are emitted either at the time of pollen release or when stigmas are receptive to pollen, (2) these volatiles diffuse or are transported to locations where pollinators occur, (3) these volatiles are intercepted by pollinator sensory systems, (4) the information content in the volatiles can be decoded by innate sensory networks or networks built on associative learning based on past experience of pollinators with plant rewards and (5) pollinators utilise this information to locate the source of the volatiles and thereby the expected location of floral rewards. A successful brood-site pollination system may also require that (1) the floral structures serve as sites for pollinator mating or that mated pollinator females are attracted to the floral structures and oviposit into them during or after pollination and (2) the floral structures, which could be single flowers or inflorescences, serve as viable brood sites that support the successful development of pollinator progeny. The latter requirement may place constraints on the size and reproductive strategy of the pollinators that could engage in this type of mutualism, since pollinator progeny must be able to acquire all resources from the brood site itself. Furthermore, the time taken for progeny ontogeny must match the trajectory of development, availability or senescence of the brood site which may be a fruit in ovule/seed parasites (e.g. fig wasps, yucca moths, leafflower moths) or flowers in pollen parasites (thrips) or senescing and decomposing floral tissue (flies, cycad-pollinating beetles). It is therefore possible that a set of host plant and of pollinator traits may determine the degree of specificity between plants and pollinators in brood-site pollination mutualisms (Table 10.1).

Sakai (2002) noted that there was high specificity between pollinators and hosts in ovule/seed parasites; this may be necessitated by the requirement for synchronisation between the development times of fruit and pollinators. Such synchrony may not be necessary for pollen parasites or those developing on decaying flowers; therefore, there may be less specialisation of pollinators on such plant hosts, and individual pollinator species may use several host plant species as long as they provide brood sites and associated rewards. However, in both cases, there may be high specialisation of plants on certain groups of pollinators (Sakai 2002) that can successfully exploit these floral resources or are deceived into perceiving that such resources exist.

Pollinators in brood-site pollination mutualisms are often primarily attracted by floral scent over long distances (Hossaert-McKey et al. 2010). Floral scents encompass over 1700 compounds in several different chemical classes (Knudsen et al. 2006; Raguso 2008). The compounds that are attractive to pollinators may vary in concentration by orders of magnitude in different plant species (Schiestl 2015). Many active floral scent constituents may be preadaptations for other physiological purposes in the plant (Schiestl and Cozzolino 2008). Coupling the constraints of using an olfactory channel for communication with the constraints imposed by pollinator reproduction in a brood-site pollination system, certain predications can be made about the nature of the communication. Plants engaging in ovule-or seed-parasitic brood-site pollination systems must send out very specific volatile messages to attract particular pollinator species. Plants engaged in pollen-parasitic brood-site systems, or those in which decaying tissues are used as brood sites, may send out signals that attract certain groups of pollinators that may not be host-plant specific but more promiscuous in their affinities, e.g. cycad cone volatiles

Table 10.1 Biology of brood-site pollinators and floral scent characters

	Pollinator longevity	Adult feeding	Mating site	Sex engaged in pollination	Type of pollination	Flower filters	Larval development	Pupal development	Pollinator → plant host specificity	Plant → pollinator specificity	Other floral signals	Floral scent specificity
Fig wasps	1–2 days	No	For pollinator progeny	Only females	Active and passive	Yes (e.g. ostiole)	Yes (larvae immobile, confined to individual flower galls)	Yes	High	High	Fig surface chemistry	High
Yucca moths (*Tegeticula*)	Few days (3–5)[a]	No	Yes	Only females	Active	None?	Yes (larvae mobile, can feed on many seeds)	No	High	High	Flower colour?	High
Leafflower moths (*Epicephala*)	Few days?	Yes?	?	Only females	Active	None?	Yes (larvae mobile, can feed on many seeds)	No	High	High	Flower colour?	High
Greya moths	Few days?	Yes	No	Both	Passive	None	Yes (only early instars)	No	Variable	Not specific	Flower colour?	?
Hadena moths	30 days[b]	Yes	No	Only females?	Passive?	None	Yes	No	Variable	Not specific	Flower colour?	?
Chiastocheta flies	?	Yes	Yes	Both	Passive	Yes, sepals	Yes	No	High	High	Yes, flower colour	Yes
Cycad thrips	?	Yes	Yes	Both	Passive	?	Yes	? Probably pupate in soil below plant	High?	High?	Yes, heat	Yes

Information to construct this table is available in the general references provided in the text with a few exceptions: [a](Dodd and Linhart 1994), [b](Lloyd 1920) mediating pollination by thrips, weevils or both (Terry et al. 2004). In such cases, plants may achieve pollinator specificity and reproductive isolation by imposing morphological filters after a certain set of pollinator taxa have been attracted (Martos et al. 2015). However, *Cycadothrips chadwicki*, previously thought to pollinate up to ten *Macrozamia* cycad species in Australia within a brood-site system, is now believed to consist of a set of cryptic species each of which is more closely associated with fewer plant hosts (Brookes et al. 2015). Communication by volatiles in cycads is complicated by cone thermogenesis where heat influences the volatile emission/diffusion rate, constitutes a multimodal signal together with volatiles and functions as an independent reward (Suinyuy et al. 2013). The biology of the pollinator must also be taken into account when attempting to understand the specialisation of signals and the evolution of responses in brood-site pollination systems. For example, in bogus yucca moths, larval survivorship on host plants can affect the possibility of host shifts even though female moths may be attracted to the flowers of several yucca species (Althoff et al. 2014).

10.1.2 Scent Broadcasting in Brood-Site Pollination: Specific Examples

The volatiles produced in brood-site pollination systems have been extensively reviewed by Hossaert-McKey et al. (2010), and the intention of this chapter is to add new findings and more importantly to deal with the constraints that plants and pollinators have faced and the solutions they have come up with in evolutionary time to solve the problems of sending, receiving, interpreting and responding to these messages.

10.1.2.1 Brood-Site Scents in the Fig–Fig Wasp Mutualism

Of all brood-site pollination mutualisms, the fig–fig wasp mutualism has been most investigated from the perspective of volatile communication (van Noort et al. 1989; Grison-Pigé et al. 2002; Hossaert-McKey et al. 2010). The fig is an enclosed globular inflorescence (the syconium) with a single tight opening, the ostiole, which can serve as a mechanical filter to restrict fig wasp entry. Some fig species are protogynous and monoecious with male and female flowers in the same syconium, while others are dioecious in which male trees produce pollinators while female trees produce seeds (Cook and Rasplus 2003; Herre et al. 2008). In monoecious figs pollinator progeny develop within syconia where mating between winged female and wingless male offspring occurs. Winged females leave the natal syconium with pollen to find one in the female flower or pollen-receptive phase; pollinators locate such syconia using volatiles. Pollen-carrying wasps that enter syconia pollinate and also oviposit into some of the uniovulate flowers, which develop into galls in which larvae feed on the endosperm. Flowers that receive only pollen develop seeds. Pollinator larvae are immobile and are confined to individual galls in which they complete their entire development until eclosion. In such a brood site, there is coordination between seed maturation and pollinator development and also clock gene-based regulation of pollinator eclosion such that males eclose first (Gu et al. 2014) to release females from their galls for mating. There are complex feedback loops between the myriad syconium inhabitants that may also comprise several parasitic fig wasp species (Borges 2015), and these feedbacks influence the tempo of brood-site development (Krishnan and Borges 2014).

The fig system is probably the only brood-site pollination system where the penalty for making mistakes can be as high as zero reproductive success for a wasp that enters the wrong host. This is because fig wasps often lose their wings and parts of their antennae during passage through the tightly fitting ostiolar opening of the syconium. Having entered the fig, the pollinator must be able to gall flowers to provide nutrition for its offspring. Therefore, there should be tight correspondence between the floral scent messages emitted by the syconia, those received by the pollinator and the ability to gall fig flowers within these syconia, since this ability is an important limiting factor for fig wasp reproduction (Ghana et al. 2015). Furthermore, pollinator lifespans range from 24 to 48 h (Ghara and Borges 2010), so host fig species with syconia in the pollen-receptive stage must be found within this short window of time. The close species specificity observed by Sakai (2002) in pollinating ovule/seed parasites and their host plants must derive in part from the high penalty for making mistakes as a result of the developmental constraints faced by pollinators in brood sites (Table 10.1). Also, since the offspring of pollinating fig wasps only mate within the syconium, the brood site is also the mating site for the next generation of pollinators; thus, there is an added selection pressure to enter the ‘right’ fig as mistakes could doom offspring to zero reproductive success. It is true, however, that since fig wasps have a haplodiploid breeding system, mated female fig wasps could lay either unfertilised haploid eggs resulting in male offspring or fertilised diploid eggs leading to females. Consequently, a single mated female wasp within the ‘wrong’ fig could at least ensure brother–sister matings. Should this breeding system encourage promiscuity in attraction to host fig species? In a set of six dioecious species, only 1.5 % of pollinators that entered the figs were the wrong species (Moe et al. 2011). Interestingly, while several fig species exhibit introgression of genes and hybridisation (Machado et al. 2005; Moe and Weiblen 2012; Wei et al. 2014), possibly resulting from pollinators entering unusual hosts, pollinators themselves constitute tighter genetic lineages and exhibit greater reproductive isolation (Machado et al. 2005). This may also result from the high amount of inbreeding that may occur in fig wasps (Askew 1968). Such mating restrictions can influence the repertoires of olfactory receptor (OR) genes and thereby the evolution of olfactory specialisation towards certain syconial scents. Therefore, the mating and developmental requirements of pollinators may be mostly responsible for keeping fig gene pools isolated. On the other hand, fig species that share pollinators may prevent large-scale genetic introgression and hybridisation by ecological factors such as differences in flowering phenologies and geographical isolation (Wei et al. 2014). Whether the floral scents of this fig species complex that shares pollinators could facilitate the breakdown of scent-mediated prezygotic barriers to hybridisation is unknown.

In dioecious fig species, pollinators can only breed within male trees, and female trees produce only seeds (Cook and Rasplus 2003; Herre et al. 2008); this means that pollinators entering syconia on female trees doom their lineages to extinction since they are unable to breed within them. The cause of this fatal attraction is believed to be intersexual mimicry in volatile signals between the sexes such that pollinators leaving male trees are unable to distinguish between a pollen-receptive female tree and a male tree (Soler et al. 2012) and are thereby tricked into entering the unrewarding and fatally deceptive syconia on female trees (Grafen and Godfray 1991; Hossaert-McKey et al. 2016). Consequently, chemical mimicry between the sexes in dioecious species is tighter when both sexes flower at the same time (Hossaert-McKey et al. 2016). In dioecious species also, the constraints on host specificity imposed by the requirement for pollinator mating sites and successful development of pollinator progeny apply, and these constraints must exert considerable pressure on the evolution of the specificity of the interaction.

If there is a requirement for such compatibility between figs and pollinators based on constraints brought about by pollinator life histories, are pollen-receptive (floral) volatile signatures unique for each fig species? Most fig species do emit a species-specific scent (Hossaert-McKey et al. 2010; Borges et al. 2013). The scent glands are present on ostiolar bracts lining the opening of the syconium and in the epidermal and subepidermal tissues on the syconium surface (Souza et al. 2015). Therefore, the fig system appears to be one in which extrafloral structures in proximity to the exterior are involved in scent production and pollinator attraction. This is suitable for a system in which the flowers are enclosed within the syconium receptacle.

While the fig–fig wasp system is a remarkable instance of plant–pollinator co-diversification (Cruaud et al. 2012), there are several examples of breakdown in specificity. About 30 % of fig species are associated with more than one species of sympatric pollinator (Yang et al. 2014), and pollinator wasp species that utilise more than one fig species are known (Erasmus et al. 2007; Moe et al. 2011; Cornille et al. 2012; Liu et al. 2015). In one of these cases, the scents of the two fig species that are visited by the same pollinator are similar (Cornille et al. 2012); whether this applies for the other examples is not yet known. There are examples of the same individual fig tree hosting several species of pollinating fig wasps and even the same syconium hosting more than one species of pollinator at the same time (Compton et al. 2009; Conchou et al. 2014). The mechanisms behind such co-occurrences of pollinators are unknown, but Conchou et al. (2014) suggest that diel variation in fig syconium scent (Borges et al. 2013; Conchou et al. 2014) may be responsible for the arrival of two co-occurring pollinators at different times.

Two or more pollinators (co-pollinators) are as likely to occur in monoecious figs as in dioecious figs (Yang et al. 2014). By conducting experimental introductions of pollinators, Moe and Weiblen (2012) suggested that the fitness costs of making mistakes and entering the wrong fig species are high in dioecious figs and attributed this to the inability of pollinators to develop in the wrong fig hosts. The fitness cost of entering the wrong fig has not been rigorously measured for monoecious figs (Yang et al. 2014) and merits investigation. Co-pollinators in monoecious figs were either sister species or unrelated species, while those in dioecious species were always sister species (Yang et al. 2014). From the host fig perspective, Proffit and Johnson (2009) found that the scent profiles of two closely related species, *Ficus sur* and *Ficus sycomorus*, were quite different. Since sister species of pollinators are likely to have more closely related olfactory systems and closely related fig species may have greater similarity in syconial scents, the role of relatedness between host signals and between receivers in the co-diversification between figs and their pollinators may show patterns that can be predicted. However, the mechanisms involved in co-speciation or host switches are complex (Machado et al. 2005) and need careful examination.

The requirement for specificity between figs and their pollinators dictated by pollinator biology has led to the suggestion of private olfactory channels for communication between figs and pollinators, e.g. the use of 4-methylanisole that makes up almost 98 % of the scent of receptive syconia in the dioecious *Ficus semicordata* (Chen et al. 2009; Soler et al. 2010). While 4-methylanisole has not been found in other fig species whose scents have been examined (Chen et al. 2009), it occurs in at least 17 angiosperm and two gymnosperm families (Schiestl 2010) and could therefore be available in the general background odours in a landscape where figs occur. Moreover, Wang et al. (2013) found that the pollinator of *F. semicordata* was also attracted by other volatiles produced by a sympatric fig variety usually pollinated by another fig wasp species and concluded that contact cues from the surface of the fig syconium are likely to be the deciding factor in reproductive isolation in this instance. While both wasp species could enter and oviposit in both fig varieties, fig syconia receiving hetero- or extra-varietal pollen were generally aborted and produced fewer seeds. Despite the viability of the hybrid seeds and their normal germination, reduced seed production can make it important for figs to ensure pollinator fidelity. Private channels may, however, not be generally employed in the fig system to ensure pollinator fidelity. Instead, figs appear to emit floral scents whose constituents are widely available in other plant species.

Another important feature of the biology of fig–pollinator mutualisms that may impact the specificity of syconial signals is that some species engage in active pollination (Cook and Rasplus 2003). Actively pollinating females have mesothoracic pockets into which they collect pollen that is deliberately unloaded onto the stigmas during pollination. Other fig species engage in passive pollination and produce massive amounts of pollen that adhere to pollinator bodies. Whether the specificity of fig scents and of pollinators are greater in actively pollinated systems compared to passive systems is unknown but should be investigated.

Fig wasp pollinators are wind dispersed and may carry pollen over distances up to 160 km (Zavodna et al. 2005; Ahmed et al. 2009). Indeed, monoecious fig wasps often exhibit weak population genetic structure over wide areas, reflecting such wide-ranging movement (Kobmoo et al. 2010). Dioecious fig species, on the other hand, tend to have more structured genetic populations (Dev et al. 2011; Nazareno et al. 2013) probably due to denser fig populations and shorter movements of their pollinators. Yet, at a continental scale (India, China and Thailand), significant geographical variation in floral scent was observed in two widely distributed fig species, one monoecious and the other dioecious (Soler et al. 2011), suggesting that local factors influence syconial scent. This is difficult to reconcile for the monoecious fig species, especially since its pollinating wasp exhibits genetic homogeneity over much of South-east Asia, suggesting wide-ranging gene dispersion (Kobmoo et al. 2010). However, since the active ingredients relevant to pollinators within the syconial signature are unknown, it is possible that the statistically significant variation observed in syconial scent may be due to variation in components that are not necessary for the host species recognition signal.

10.1.2.2 Scents in Other Brood-Site Seed-Parasitic Pollination Mutualisms

There are several other classic cases of brood-site seed-parasitic pollination mutualisms that have begun to be investigated from the perspective of floral scent (Table 10.1). These include interactions between yuccas (*Yucca*) and yucca moths (*Tegeticula*); leafflowers (*Glochidion*) and leafflower moths (*Epicephala*); and the globeflower (*Trollius europaeus*) and anthomyiid flies (*Chiastocheta*). In these seed-parasitic mutualisms, the penalty for making mistakes is not as high as in the fig system. Does this mean that floral scent specificity is lower in such mutualisms compared to the fig system? As in some fig wasps, female yucca and leafflower moths also have specialised anatomical modifications to collect pollen (maxillary tentacles in yucca moths: Pellmyr 1997; Pellmyr and Krenn 2002; specialised proboscis hairs in leafflower moths: Kawakita and Kato 2006) and use these structures to engage in active pollination. The evolution of such innovative structures to facilitate active pollination should indicate high interaction specificity and may also suggest why active pollination is uncommon in insect pollinator lineages (Pellmyr 1997). In several mutualisms where pollinator moth larvae consume seeds [e.g. *Glochidion* (Okamoto et al. 2007) and *Breynia* pollinated by *Epicephala* moths (Svensson et al. 2010)], the floral scent attractive to females is composed of compounds that are part of the scent repertoire of several other plant species, suggesting a lack of private channels. However, there were statistically significant differences between the scents of different *Glochidion* species (Okamoto et al. 2007) suggesting specificity. In the interaction between *Yucca* and its mutualistic *Tegeticula* moths, floral scent across several allopatric *Yucca* species comprised commonly available floral scent compounds but was very similar between species, suggesting that scent composition is conserved in yuccas of section Chaenocarpa (Svensson et al. 2006, 2011). The geographical isolation of yucca species (Althoff et al. 2012) may contribute to this scent conservatism.

In another seed-parasitic system involving *Greya* moth species that pollinate several geographically isolated *Lithophragma* species, floral scent is an important pollinator attractant (Friberg et al. 2014). Here, the different species of *Lithophragma* have divergent floral scents (Friberg et al. 2013), which may contribute to their reproductive isolation while in sympatry, even though they are serviced by the same moth species. However, in this system, multiple traits such as floral morphology and local variation in moth morphometrics and oviposition behaviour may also contribute to reproductive isolation between plant species and to the coevolution between plants and their pollinators (Thompson et al. 2013). In systems involving *Greya* and *Hadena* moths, host plants are also serviced by other pollinator taxa such as bees (Kephart et al. 2006) (Table 10.1); hence the specificity of the floral scents in attracting pollinators is questionable in these systems.

In the highly specific nursery pollination system involving the globeflower *Trollius europaeus* and *Chiastocheta* flies, six out of 16 compounds commonly found in the scents of freshly bloomed flowers are detected by the fly antenna (Ibanez et al. 2010). Furthermore, these active compounds showed less variation than other components of the floral scent, suggesting stronger selection on their emission through pollinator perception. In the *Trollius* system, as in figs, morphological filters, i.e. space between sepals, may serve to increase the specificity of floral visitors (Ibanez et al. 2009a). Flies use the closed *Trollius* flower as a mating site where males and females feed on nectar and both sexes engage in passive pollination (Després 2003). Several species of pollinating *Chiastocheta* flies arrive in sequence to oviposit early or late in the life of the single multicarpellate flower produced per plant. The earliest-arriving fly species is also the most mutualistic, and lays only a single egg in each flower, while later-arriving species deposit multiple eggs per flower (Després and Jaeger 1999). Since an individual flower receives pollen and pollinator eggs over several days, it is possible that the scent of the globeflower later in its life is mixed with herbivore-induced plant volatiles (HIPVs), resulting from feeding by early-arriving pollinator larvae, or with oviposition-induced plant volatiles (OIPVs) (Fatouros et al. 2012) resulting from a reaction to pollinator eggs. HIPVs and OIPVs may be part of the scent mixture that attracts later-arriving pollinator species. Globeflowers produce an induced chemical defence in response to egg deposition by pollinating flies, and this may help to control overexploitation of the carpellary brood sites by later-arriving pollinators (Ibanez et al. 2009b). It is not inconceivable that HIPVs and OIPVs contribute to a combined process of attraction and repulsion as suggested in figs (Borges et al. 2013; Borges 2015).

In another newly discovered seed-parasitic brood-site pollination mutualism, the fungus gnat *Bradysia* is attracted to 2-methyl butyric acid methyl ester produced by *Rheum nobile* flowers (Polygonaceae) (Song et al. 2014). Since this plant also sets seed by autonomous self-pollination and the specificity of this interaction is not yet known, further studies are awaited.

10.1.2.3 Brood-Site Mimicry Pollination Systems

In such systems, plants deceive pollinators by signalling the availability of brood sites when none actually exist. The scent production in these systems has been reviewed recently (Vereecken and McNeil 2010; Urru et al. 2011; Jürgens and Shuttleworth 2015) and will not be dealt with in detail in this chapter. There are three main pollination systems in which flowers mimic the scent and other features associated with oviposition sites: sapromyophily (involving dung and carrion flies), coprocantharophily (attracting dung beetles) and mycetophily (utilising fungus gnats). In these systems, floral scent is extremely important in attracting pollinators, although visual and thermal signals may also be important. In such systems, pollinators do not usually oviposit into the flowers since additional short range cues that release egg-laying behaviour in real brood sites are missing (Vereecken and McNeil 2010; Urru et al. 2011). As in other mimicry systems, it is possible that frequency-dependent selection operates on the abundance of these flowers/plants such that these fraudulent structures have limited spatiotemporal representation in their habitats. However, since these pollination systems depend on the sensory exploitation of pathways that govern the innate attraction of pollinators to certain compounds, strict frequency-dependent constraints may be lifted on these mimics (Jürgens and Shuttleworth 2015).

10.2 Reception of Floral Volatiles by Pollinators

All pollinators in brood-site pollination mutualisms are insects, mostly Hymenoptera, Lepidoptera, Coleoptera, Diptera and Thysanoptera. Since there is very little knowledge of the olfactory systems of pollinators engaged in brood-site pollination mutualisms, the following sections will use principles of odour reception and detection that have been gleaned from studies on model organisms such as *Drosophila*, *Manduca sexta* and *Apis mellifera*. Since these are representatives of the Diptera, Lepidoptera and Hymenoptera that are so important in brood-site pollination, it is hoped that the general principles these models provide will apply to other pollinator representatives of these taxa. Also, since most insects associated with brood-site pollination mutualisms are relatively short-lived and specialised (Table 10.1), it is assumed that learning is not important in the host location process; consequently, the interaction between associative learning and olfaction-based host location has been ignored in this chapter.

10.2.1 Signals, Cues and Pre-existing Biases

Floral volatiles may have arisen as by-products of physiological processes in plants (Peñuelas and Llusià 2004); this is especially true for protective chemicals, which later acquired signal content (Steiger et al. 2010). This may explain the presence of many common volatiles in floral scents across plant families. Floral volatiles may have also evolved as a compromise between signalling to attract pollinators and third-party mutualists such as parasitoids and to repel unwanted visitors such as florivores or herbivores. Thus floral scent inevitably incorporates volatiles resulting from selection on the whole plant (Raguso 2008, 2009; Junker and Parachnowitsch 2015; Chap. 11). Floral scent could, therefore, inadvertently provide cues to unwanted visitors that are attracted and not repelled by its constituents. Abiotic features of the environment can also cause intraspecific variation in the quality and quantity of floral scent (Majetic et al. 2009). Yet, increasing the quantity of floral scent may not necessarily increase attraction as shown for *Manduca sexta*, which exhibited the same behavioural response to a 1000-fold variation in floral scent concentration (Riffell et al. 2009). Therefore, how much floral scent a plant should optimally produce is not well understood.

Floral scents may incorporate true evolutionary novelties and convergences onto scent components relevant to pollinators, such as prey alarm pheromones (Stökl et al. 2011), green leaf volatiles that signal herbivorous prey to predatory wasps (Brodmann et al. 2008), mimics of pollinator sex pheromones (Peakall and Whitehead 2014), deceptive scents that signal availability of food to kleptoparasitic pollinators (Heiduk et al. 2015, Oelschlägel et al. 2015), the scent of brood sites involving mimicry of carrion odours attracting flies, dung/urine odours attracting beetles and flies and the fly-attracting odours of fermenting fruit (Urru et al. 2011; Moré et al. 2013). In floral scents that mimic vital features of insect biology such as food resources or mates, even imperfect mimicry is adequate to attract pollinators by capitalising on sensory exploitation (Schaefer and Ruxton 2009). Tapping into pre-existing olfactory circuits can effectively draw flies to pollinate flowers that mimic the odour of yeasts (Stökl et al. 2010) or may be responsible for the convergence of moth-pollinated flowers towards certain scent combinations (Riffell 2011). Why moths have pre-existing circuits that are tuned to oxygenated aromatic compounds that dominate the scent of many moth-pollinated plants (Riffell et al. 2013) is not known; however, aromatic compounds are also emitted by many insect orders including Lepidoptera (Schiestl 2010). It is possible, therefore, that moth-pollinated plants emit volatiles that moths also employ in their own communication and hence exploit pre-existing biases in moth receivers to facilitate this cross-kingdom communication (Schiestl 2010).

10.2.2 Chemical Noise amid Floral Volatile Plumes

The airspace contains many volatiles of biogenic origin that also exhibit diel and seasonal changes (Laothawornkitkul et al. 2009; Jardine et al. 2015; Yáñez-Serrano et al. 2015). How pollinators deal with this background chemical noise considering that most floral scents are composed of a common set of compounds is one of the most challenging aspects of volatile reception and behavioural response (Schröder and Hilker 2008; Nehring et al. 2013; Wilson et al. 2015) and is the olfactory equivalent of the acoustic cocktail party effect (Krishnan et al. 2014) (Fig. 10.1). The insect pollinator olfactory detection and reaction system is hierarchical (Wicher 2015). The peripheral detection system consists of olfactory receptors (ORs) borne on olfactory receptor neurons (ORNs) housed in porous protuberances (sensilla) on the antenna. ORNs bearing similar ORs converge onto hubs or nodes (glomeruli) in the antennal lobe, and from here information is transferred to higher

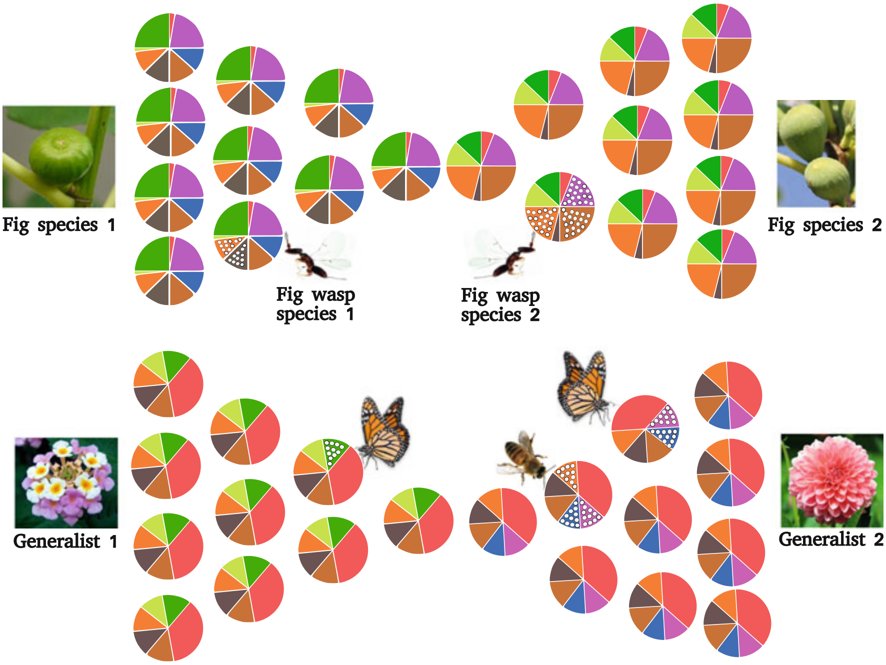

Fig. 10.1 Plants emit volatiles from flowers or associated structures to attract pollinators. Volatiles are emitted into the air and move as packets depicted here as pie charts, where each colour denotes a different volatile. *Dotted colours* indicate volatiles that are not detected by pollinator antennae. Though specialist plants such as fig species (1 and 2) emit many volatiles in common, they may do so in different ratios, and their specialist fig wasp pollinators (species 1 and 2) may not detect all compounds. This may confer specificity in the interaction between plants and pollinators. Generalist plants (1 and 2) may also share emitted volatiles and, if pollinated by the same species, may attract them using the same set of volatiles or a subset of these compounds based on what the antennae detect

processing centres such as the mushroom body or lateral protocerebrum where behavioural decisions are made (Szyszka and Galizia 2015) (Fig. 10.2).

Floral scents emitted from plants spread by the slow process of molecular diffusion and the faster process of advection via windy airflows which are often turbulent (Weissburg 2000). Scents move in filaments consisting of packets of high odour concentration interspersed with areas in which the odour is absent (Murlis et al. 1992). This means that a pollinator searching for a relevant floral odour could perform anemotaxis (using surging upwind flight). However, since the odour is intermittently available, many pollinators perform a casting or zigzagging behaviour when they first encounter an odour plume with some valence (i.e. behavioural relevance) in order to increase the probability of sustained encounter with the scent (Vickers 2000; Cardé and Willis 2008; Beyaert and Hilker 2013). Some insects use wing movements to actively draw air over the antennae (Sane and Jacobson 2006), which might effectively increase the encounter of antennal sensilla with floral scent compounds.

Wind speeds and the structures of odour plumes at the locations where pollinators encounter them will determine their responses and their ability to track floral resources. For example, the wind-dispersed pollinators of fig trees were captured throughout the diel cycle at heights ranging from 25 to 75 m above the ground in tropical forests in Asia where the canopy is about 35 m in height (Harrison and Rasplus 2006). Pollinators of dioecious figs may, however, be found at lower heights compared with those of monoecious figs (Harrison 2003); at these heights wind speeds are lower, and the scent plume structure and movement is likely quite different (Girling et al. 2013). Thrips that are important in brood-site pollination mutualisms are also wind dispersed (Appanah and Chan 1981). How fig wasps and thrips respond to odour plumes at the heights at which they travel is scarcely known. Since plumes are dynamic, the olfactory detection ability of pollinators must match this spatiotemporal variability. This is why the initial process of olfaction in insects is fast; olfactory transduction can occur within 2 ms and fluctuating odour stimuli can be resolved at frequencies of more than 100 Hz at the antenna (Szyszka et al. 2012, 2014).

10.2.3 Reception and Decoding of Volatile Messages

Although hundreds of volatiles may occur within a floral scent, the pollinator antenna can detect only a subset of these (Bruce et al. 2005) (Figs. 10.1 and 10.2). The detection can be influenced at a variety of levels.

10.2.3.1 Discrimination at the Olfactory Receptor Level

The first possible filter to 'noise' in the floral signal is the presence of odorant-binding proteins (OBPs) in the peri-sensillar space (Fig. 10.2); these OBPs are

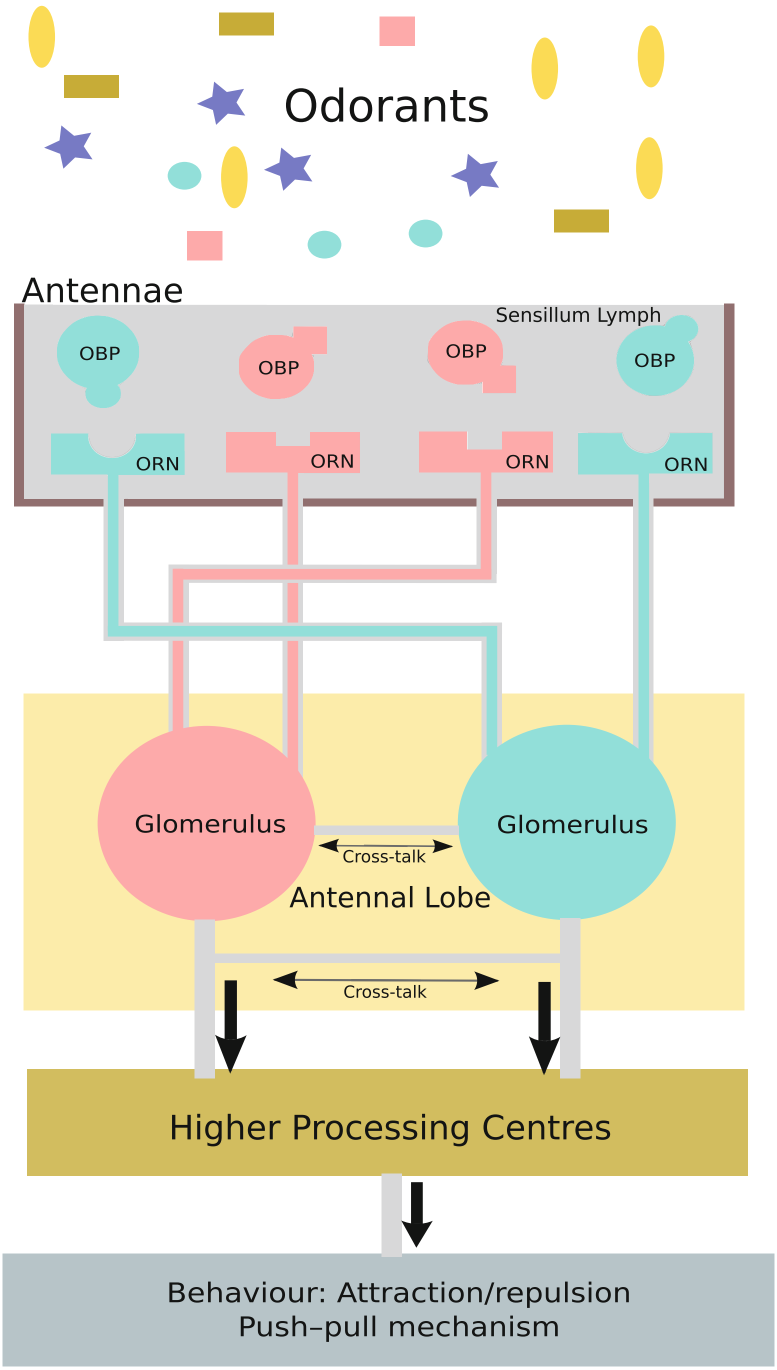

Fig. 10.2 Schematic depicting the flow of information from the peripheral olfactory detection system at the insect antenna to the higher processing centres culminating in attraction or repulsion behaviour in response to volatile reception and crosstalk (excitation or inhibition) between olfactory circuits at various levels. The figure is not drawn to scale and omits anatomical details.

highly diverse and may help in the transport of highly hydrophobic volatiles into the OR detection system (de Bruyne and Baker 2008). The fig wasp *Ceratosolen solmsi* that pollinates the dioecious *Ficus hispida* has only seven OBPs. This is one of the smallest insect OBP repertoires and may reflect its high host specificity (Wang et al. 2014). ORs show high rates of evolutionary change (McBride and Arguello 2007; Nei et al. 2008; Ramdya and Benton 2010; Cande et al. 2013). Compared to humans in which there are 800 OR genes and ~400 intact ORs (Mainland et al. 2014), insects have fewer OR genes, e.g. 163 in *Apis mellifera* (Robertson and Wanner 2006), 62 in *Drosophila melanogaster* (Robertson et al. 2003) and 79 in *Anopheles gambiae* (Hill et al. 2002). These numbers have been further reduced in the highly specialised fig wasp *C. solmsi* which has only 46 OR genes (Xiao et al. 2013) some of which are evolving rapidly (Lu et al. 2009). Similarly, specialist flies lose OR genes ten times faster than related generalist species (McBride 2007).

Despite the relatively small number of ORs, pollinators use volatiles to navigate and find their mates, resources and brood sites in a world of great volatile complexity. Much of the discrimination could occur at the peripheral olfactory system (Hansson and Stensmyr 2011; Bohbot and Pitts 2015). Most ORs are believed to harbour multiple sites where portions of odorants can bind; a combination of odorants binding at different receptors can trigger a seemingly endless set of coding combinations via a combinatorial code for odour detection (Malnic et al. 1999). While many receptors respond to a wide range of ligands (volatiles) at high concentrations (e.g. at the micromolar scale), they are actually narrowly tuned to only one ligand at lower concentrations (e.g. at the nanomolar scale) (Hughes et al. 2010; Liu et al. 2014; Bohbot and Pitts 2015); it is possible that such low concentrations have real ecological relevance in scent plumes (Andersson et al. 2015). Bohbot and Pitts (2015) plead for greater understanding of receptor binding and the search for ligands with ecological/behavioural valence at biologically relevant concentrations. This is why knowledge of 'real' concentrations of floral scent volatiles within 'real' plumes becomes so important in understanding host localisation by pollinators.

10.2.3.2 Discrimination at the Sensillum Level

Since pollinators encounter scent plumes with high intermittency of odour presentation owing to their dynamic nature in turbulent airflows, it is important that sensilla contain ORNs that are tuned to those components in the relevant mixture that are most likely to be found together (i.e. components of a host plant floral scent) compared within another odour plume in its vicinity. Binyameen et al. (2014)

Fig. 10.2 (continued) OBP odorant-binding protein in the peri-sensillar space; *ORN* olfactory receptor neuron. ORNs that bind to one type of odorant converge onto one type of glomerulus showed that co-localisation of sensilla improved the spatiotemporal resolution of odours. Other workers have hypothesised that co-localisation of specific ORNs in sensilla may help detect ratios of ecologically relevant scents (Bruce et al. 2005). Often ORNs within the same sensillum may be tuned to volatiles within different biosynthetic pathways, and this may also give pollinators an additional level of discrimination about the source and valence of the volatiles (Bruce and Pickett 2011).

10.2.3.3 Discrimination at the Glomerulus Level and Higher

Within the antennal lobe, local neurons play an important role in inhibition, excitation and synergistic effects within and between glomeruli (Fig. 10.2). Output projection neurons convey this summated information to the mushroom bodies and to the lateral protocerebrum where higher-order processing of olfactory information occurs (Galizia 2014). Push–pull circuits must operate in the antennal lobe via exhibitory–inhibitory connections that exist between neurons and discrimination of scents may be fine-tuned by such interneuron activity (Cunningham 2012; Galizia 2014). However, in some insects, those glomeruli housing neurons that have narrowly tuned ORs tend to have fewer local interneurons (Chou et al. 2010) suggesting that they belong to dedicated circuits that do not require lateral inhibition (Galizia 2014). Only comparison between circuits in pollinator species that have different ecologies such as specialist versus generalist pollinators will suggest general design principles (see Burger et al. 2013 for such a comparison between an oligolectic bee and the generalist honeybee).

10.2.3.4 Co-ordinated Changes at Different Sensory Levels to Match Ecology and Physiology

The olfactory system can solve problems of reception and interpretation of volatile messages in various ways. In specialist insects, certain ORNs and corresponding glomeruli can be over-represented at the cost of others to improve detection and host localisation (Stensmyr et al. 2003; Dekker et al. 2006; Linz et al. 2013; Goldman-Huertas et al. 2015). If ORNs do not vary in number and type across insect lineages, they can vary in sensitivity and temporal firing pattern in response to preferred host volatiles (Olsson et al. 2006a, b) in order to solve the detection problem. Some volatiles in the scent blend may evoke a stronger positive response than volatiles tested alone, suggesting synergistic effects (Tasin et al. 2007). Some insects show the same behavioural response to the whole blend and to a subset of volatiles in the blend suggesting redundancy in volatile messages (Tasin et al. 2007). It is extremely important to investigate such information redundancy, especially in the context of atmospheric pollution that can degrade floral volatiles and interfere with the interpretation of floral messages by pollinators (Lusebrink et al. 2015).

Since (1) maintaining sensory tissue is expensive, selection for pruning unnecessary sensory circuits should occur (Niven and Laughlin 2008), (2) many species exhibit an over-representation of those ORs/ORNs/glomeruli pertinent to host ecology at the expense of irrelevant circuits indicating that such pruning does happen in the olfactory system, and (3) most insect species in brood-site pollination systems are small and have special life history constraints, it is tempting to speculate that such species may not be able to support generalised 'jack-of-all-trades' types of olfactory systems, and hence specialisation will be the norm.

Another way to increase the appropriateness of pollinator behavioural responses to volatile signals is to have the olfactory system modulated by physiological events such as mating. In male and female moths, responsiveness of antennal lobe glomeruli is modulated after mating, resulting in greater attraction towards volatiles of food resources or of oviposition substrates, respectively (Saveer et al. 2012; Kromann et al. 2014).

10.2.4 Push–Pull Mechanisms in a Sea of Volatiles

If pollinators encounter volatiles in different packets, and with differences in valence (some that signify food resources, while others signify toxins, predators or other ecological attributes), how might these differences guide their behaviour so that appropriate decisions are made? In the monoecious *Ficus curtipes*, pollinating fig wasps were most attracted by the scent of pollen-receptive female syconia and were actively repelled by the scent of male phase syconia from which wasps were dispersing (Gu et al. 2012). It is believed that this change in floral scents actively repels pollen-carrying pollinators that are leaving their natal figs from remaining in the same tree, thus avoiding geitonogamy (pollen delivery in the same tree), in case pollen-receptive figs are present at the same time on the natal tree (Gu et al. 2012). In *Macrozamia* cycads pollinated by specialist *Cycadothrips*, the thrips are attracted and then repelled by altered fractions of volatiles such that they move between male and female cones to effect pollination (Terry et al. 2007); however push–pull systems do not apply to all cycads (Suinyuy et al. 2013). In a highly specialised orchid system, male andrenid bees effect pollination by pseudocopulation with the sexually deceptive flowers; post-pollination production of farnesyl hexanoate by these flowers serves as a repellent to these bees since it serves as an antiaphrodisiac (Schiestl and Ayasse 2001). A pollinating moth in a brood-site pollination mutualism also avoids flowers infected by a pathogenic fungus as this would prevent successful development of seeds and of the seed-eating larvae (Biere and Honders 2006); however, whether fungal volatiles alone are responsible for this avoidance behaviour or whether the infected plants produced an altered volatile profile is not known.

Repellency plays an important role in push–pull systems. In this case, insects may also have receptors for volatiles produced by their non-host plants so that they can recognise and avoid them (Nottingham et al. 1991). Repellent odours affect avoidance behaviour in several ways. Repellents may act at the peripheral olfactory system by serving as competitive or non-competitive antagonists of ORs or co-receptor subunits (Chen and Luetje 2013; Dickens and Bohbot 2013; DeGennaro 2015) and thus inhibit and mask the effect of a cognate ligand. Repellents may bind with the odorant and prevent its binding to the OR (Dickens and Bohbot 2013). They may act as molecular confusers by changing the response of several ORNs to the odorant and effectively scrambling odour coding by glomeruli (Pellegrino et al. 2011; DeGennaro 2015). Some repellents activate unique neural circuits dedicated to the avoidance response (Suh et al. 2004; Stensmyr et al. 2012). Repellents may carry messages about host unsuitability. For example, methyl jasmonate is an HIPV and is produced by stressed plants in which defence machinery has been activated (Schuman and Baldwin 2012). Herbivorous insects such as seed parasites in brood-site pollination mutualisms or mosquitoes in which nectar-feeding occurs (Zhou et al. 2014) should have innate avoidance behaviours to such stressed plants unless they use HIPVs to locate hosts as previously suggested. It is therefore interesting that the evolutionary origin and ecological significance of the action of the mosquito repellent *N*,*N*-diethyl-*meta*-toluamide (DEET) is believed to be due to its similarity in action and binding to the receptor for the plant volatile methyl jasmonate (Xu et al. 2014).

While individual volatiles may be repellent when presented singly, a blend containing the same volatiles may be attractive (Webster et al. 2010; Bruce and Pickett 2011) suggesting that the blend provides the appropriate behavioural context. Robust information coding systems that can distinguish host from non-host must also have the ability to cope with the prevalent intraspecific variation in host volatile profiles. Despite the importance of understanding odour repellency in host plant location, the phenomenon is not well understood.

10.3 An Evolutionary Perspective on Floral Scent in Brood-Site Pollination Mutualism: Putting It All Together

Some authors have suggested that an information theory approach could be useful in understanding volatile communication between plants and insects (Wilson et al. 2015). For such an approach, the detection of true patterns and the discovery of real processes are essential. It is clear that while common principles of volatile detection apply across systems, insects have solved the problem of locating their hosts in a variety of ways. Yet, it is important to remember that volatile communication using floral scents is a cross-kingdom communication. Since this communication signal has to work across kingdoms, from a signal fidelity point of view, i.e. being able to produce the same signal that evokes the same response in a receiver, it may be important for plants to co-opt reliable volatile production pathways that were already in existence for other basic functions; this may reduce the efficiency cost of communication (Hackett et al. 2014). Could this explain why the same sets of common volatiles/volatile classes occur repeatedly in floral scents? The onus is then on the receiver to be able to extract the signal from the 'noise' and to even determine what the signal is; it is entirely possible that the same set of volatiles presented to two different receivers would be perceived as different signals (Kessler et al. 2013) (Fig. 10.1). This is why we need more studies such as Ayasse et al. (2000), Salzmann et al. (2007) and Ibanez et al. (2010) who were able to show that the subset of floral scent compounds active at the pollinator's antenna exhibited much less variation in the blend than those to which the antenna was insensitive. Blends at ecologically relevant concentrations need to be used in all tests and even in single sensillum recordings. We also need to measure volatile fluxes at the antenna in order to understand the concentrations at which this communication occurs (Andersson et al. 2012).

The type of behavioural assay used to determine valence of compounds and blends may also influence the results. For example, Wang et al. (2013) rightly point out that many fig wasps do not perform in conventional Y-tube olfactometers with dimensions that preclude wasps from performing normal flight, so interpreting results using such setups could be questionable. In *Drosophila*, for example, there is one pathway that can trigger aversion to CO_2 in walking flies but another that results in positive chemotaxis to CO_2 when flies are in flight (Mansourian and Stensmyr 2015). Clearly, functionally relevant assay systems must be employed.

Brood-site pollination mutualisms usually exhibit a high degree of specificity between plants and individual pollinator species or groups. Many of these insects also engage in active pollination, which should indicate tight specialisation between plants and pollinators. However, it may be useful to ask whether passive pollination necessarily means lower specificity in floral scents. This may be true in pollination by *Hadena* and *Greya* moths (Table 10.1) that also use other plant hosts, but may not be true of other brood-site pollination systems. It may also be useful to examine whether the occurrence of mating at the brood site increases the specificity of the interaction. In fig wasps, mating in the next generation (i.e. pollinator progeny) occurs within the brood sites, but in other species mating of pollinators occurs on or in the brood site (Table 10.1). Is it possible that some of the components of the mating pheromone or those that release the mating sequence may also be found in the floral scent of such systems?

In this chapter, the role of factors other than pollinator attraction in altering the compositions of floral scent has not been reviewed. These factors may include biotic stresses such as deterrence of florivores or pollen and nectar thieves (Kessler et al. 2013), as well as abiotic stresses, especially under the scenario of climate change and increases in tropospheric ozone and other atmospheric pollutants (McFrederick et al. 2008; Farré-Armengol et al. 2013; Blande et al. 2014). We need to address floral scents in the context of multimodal signals (Junker and Parachnowitsch 2015). For example, even classical studies, in which visual signals coupled with morphology were considered sufficient to explain differential pollinator visitation, now also invoke the role of floral scent in promoting reproductive isolation (Byers et al. 2014a, b). It is insufficient to consider floral scents alone, even in highly specialised interactions (Rakosy et al. 2012).

We need perspectives from ecology, evolution, chemistry, atmospheric science, fluid dynamics, behaviour and neurobiology in order to make sense of the communication between plants and animals using volatiles. This is especially important in systems that are as complex as brood-site pollination mutualisms in which, besides the issues of attracting a pollinator and exchanging an ephemeral reward, there are a set of pollinator developmental and life history constraints that must be factored into our understanding. The need of the hour is collaboration between various disciplines that will provide better answers to Jean-Henri Fabre's query about a seemingly elusive communication using volatiles.

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Chapter 11
Multifunctional and Diverse Floral Scents Mediate Biotic Interactions Embedded in Communities

Robert R. Junker

Abstract Floral scent bouquets are both chemically and functionally highly diverse and are composed of volatile organic compounds (VOCs) that are attractive to pollinators, repellent to antagonistic animals and have the potential to regulate the establishment and growth of microorganisms. After briefly summarizing the biochemical basis for the diversity of floral scents, I will focus on their ecological significance in pair-wise interactions and communities. The multifunctionality of floral scent bouquets is based on the composition of VOCs that mediate one or more distinct biotic interactions. Additionally, the multifunctionality is also supported by the dynamics in emission rates of floral scents enabling flowers to synchronize with the activity patterns of pollinators in a diel rhythm and to quickly respond to biotic interactions. Finally, I will discuss how the chemical ecology of flowers can be integrated into a community ecological context where floral scents are treated as hyperdiverse functional traits involved in important ecosystem processes.

11.1 Introduction

Estimates suggest a global emission of 1000 Tg year^{-1} of volatile organic compounds (VOCs) by plants into the atmosphere (Galbally and Kirstine 2002; Guenther et al. 1995). Monoterpenes alone contribute 117 Tg year^{-1}, from which 2 Tg year^{-1} originate from flowers (Guenther 1999). Floral monoterpene emission is complemented by another 12 Tg year^{-1} of other VOCs (Guenther 1999). These numbers are even more impressive considering the relatively short flowering time of plant species as well as the small floral biomass compared to the vegetative biomass (Wardhaugh et al. 2012). Accordingly, the hourly emission of VOCs from

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flowers per unit of fresh weight strongly exceeds the emission rates of the vegetative parts in most plant species (e.g. Ashman et al. 2005; Bertoli et al. 2003) and can even be up to 6 mg h^{-1} (Dötterl et al. 2012a), suggesting a prominent role of scents in floral biology.

Flowers are the most complex and diverse structures in plants judging from their interspecific variability in morphology, coloration and volatile emissions and thus have always attracted the curiosity of researchers (Darwin 1862; Kerner 1879; Sprengel 1793). Additionally, their fascinating interactions with animals (Schaefer and Ruxton 2011; Faegri and Pijl 1979) as well as their importance for sexual plant reproduction both in natural and agricultural systems (Ollerton et al. 2011; Klein et al. 2007; Gallai et al. 2009) strongly contribute to their appeal as study subjects. Flower–animal interactions, which are a prerequisite for efficient pollination and fertilization in many plant species, are driven by the animals' interest in finding and consuming resources. Thus, flowers are forced to manipulate the behaviour of their interaction partners and thereby balance the costs and benefits for both partners (Schiestl and Johnson 2013). Floral scents have been shown to be excellent tools for immobile plants to attract animals from a distance and guide them to nutritious floral rewards, which will ideally lead to pollination (Raguso 2008b). Animals often have innate preferences for floral scents either as a result of (diffuse) co-evolution between flowers and their pollinators (Schäffler et al. 2012, 2015) or because flowers exploit pre-existing preferences for certain volatiles that are important for the animals in other contexts (Schiestl and Johnson 2013; Oelschlägel et al. 2015; Schiestl and Dötterl 2012; Heiduk et al. 2010). In generalized systems, insects often associate floral scents with rewards, which facilitates host finding and flower fidelity although these volatiles may not evoke positive responses prior to associative learning (Katzenberger et al. 2013; Wright and Schiestl 2009). In addition to the attractive properties, floral scents also have the ability to protect the reproductive structures against deleterious animals that consume rewards without providing a pollination service in return (Raguso 2008b; Junker and Blüthgen 2010). Therefore, scent bouquets often contain repellent volatiles that prevent animals from visiting flowers (Junker et al. 2011c). Likewise, floral scents have been shown to inhibit the growth of bacteria (Junker and Tholl 2013) that may be pathogens (Huang et al. 2012) or otherwise negatively interfere with pollination (Junker et al. 2014; Vannette et al. 2012). Consequently, floral scent bouquets comprise volatiles with both attractive and repellent functions (Kessler et al. 2008) and are thus important factors explaining the partitioning of flower visitors among plant species within diverse communities (Junker et al. 2010b, 2011b). Although little is known about how floral scents participate in community ecological processes, such as the establishment of flower–visitor networks, their high functional diversity suggests a significant contribution of floral scents in this context.

The functional diversity of floral scents is mirrored by the chemical diversity of VOCs emitted by flowers (Knudsen et al. 2006; Muhlemann et al. 2014). Floral scent bouquets comprise, depending on the plant species, either few or well above a hundred individual compounds that often exhibit high structural diversity even though they originate from just a handful of biochemical pathways (Knudsen and

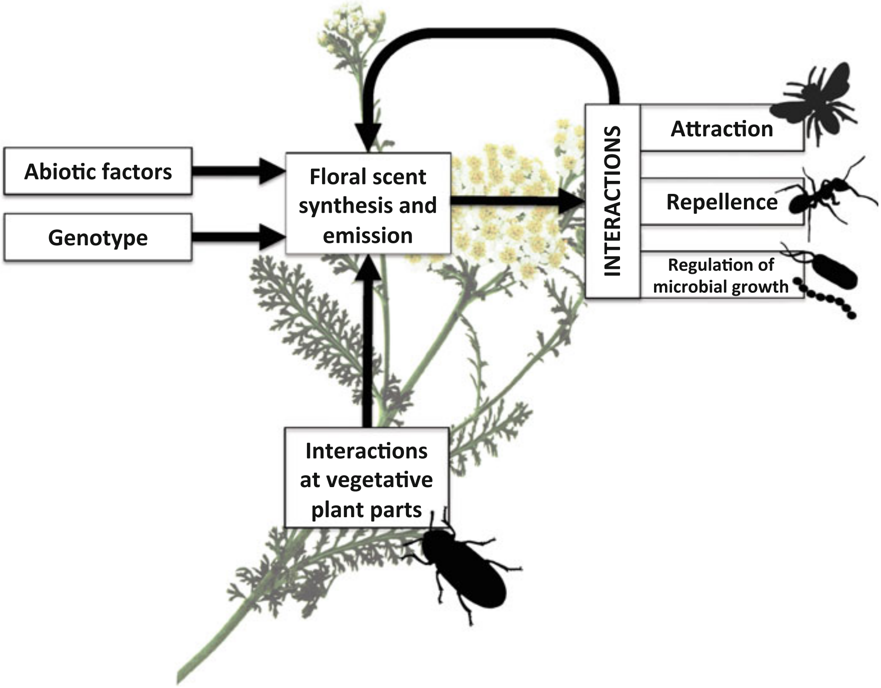


Fig. 11.1 Ecological roles of floral scents and factors explaining variation in scent emissions. Details are provided in the text. Photo courtesy of Jonas Kuppler

Gershenzon 2006). Additionally, floral scents are not a static phenotype but dynamically vary in diel rhythms (Jürgens et al. 2014), change after pollination (Schiestl et al. 1997) or respond to herbivory (Lucas-Barbosa et al. 2011).

The field of floral scent biology and related disciplines such as the neurobiology of scent perception in animals is too diverse to be covered in a single book chapter. Actually, a whole book exclusively focusing on floral scents was published a few years ago (Dudareva and Pichersky 2006a). Furthermore, a number of excellent recent review articles have covered diverse topics ranging from biochemical processes within cells (Dudareva et al. 2013) to the effects of climate change on floral scent emission (Farré-Armengol et al. 2013). After a short summary of the biochemistry of floral scent synthesis and emission, I will focus on the multiple ecological roles of floral scents and how they affect flower visiting organisms and their ability to utilize floral resources (Fig. 11.1). These aspects of floral scent biology will lead to a broader discussion on how floral scents can be treated in the context of community ecology fostering the integration of the chemical ecology of flowers into a broad ecological framework.

11.2 Biosynthesis of the Chemical Diversity of Floral Scents

Flowers usually stand out from the surrounding vegetation by their conspicuous multimodal display including bouquets of VOCs. Floral scents are produced in osmophores, glandular trichomes and hairs that are specialized organs for scent production and emission or most commonly in unspecialized epidermal cells (Effmert et al. 2006; Widhalm et al. 2015). At the subcellular level, VOCs are either produced in the cytosol or within plastids and afterwards need to be released from the cells into the atmosphere by passing the plasma membrane, the cell wall and often also the cuticle. Whereas a passive concentration-dependent diffusion is usually suggested as the mechanism of VOC release from cells, Widhalm et al. (2015) recently proposed that further active mechanisms such as vesicle trafficking or other shuttling processes are required to explain high emission rates of lipophilic VOCs through the hydrophilic cell wall.

Some VOCs such as limonene, linalool and benzaldehyde are emitted by a large number of flowering plant species, while others have been documented as emitted by a few or even a single plant species (Knudsen et al. 2006; Heiduk et al. 2015). The interspecific variability in the composition of floral scent blends is enormous due to the numerous combinatorial possibilities of frequently emitted substances and the presence of compounds unique to one or few plant species (Raguso 2008b). VOCs are derived from different biochemical pathways, and most of them belong to the monoterpenes, sesquiterpenes, aromatics and aliphatics (Knudsen et al. 2006). Less common are representatives of the diterpenes, irregular terpenes, nitrogen- and sulphur-containing compounds and miscellaneous cyclic compounds (Knudsen et al. 2006).

The methylerythritol phosphate, mevalonic acid, shikimate and lipoxygenase pathways responsible for the synthesis of monoterpenes, sesquiterpenes, aromatics and aliphatics, respectively, are well described in a number of review articles (e.g. Dudareva et al. 2013; Dudareva and Pichersky 2006a, b; Dudareva and Pichersky 2000; Schie et al. 2006; Muhlemann et al. 2014). The chemical diversity encountered in floral scents is, however, not solely the product of these 'classical' pathways even though they produce a large variety of VOCs. Many terpenes, for example, are modified after their synthesis by hydroxylation, dehydrogenation, acetylation or further types of chemical reactions (Dudareva and Pichersky 2006a). Cytochromes P450, which are hemethiolate membrane-bound enzymes that have multiple additional roles in the plants' secondary metabolism, play a prominent role in these processes (Bak et al. 2011). For instance, in *Arabidopsis thaliana* it has been shown that the multifunctional P450 CYP76C1 uses linalool (a product of a terpene synthase) as a substrate to synthesize multiple linalool oxides including 8-hydroxy, 8-oxo and 8-carboxylinalool, as well as lilac aldehydes and alcohols (Boachon et al. 2015). Other P450s are involved in the production of homoterpenes: the monooxygenase CYP82G1, expressed in *A. thaliana* flowers, catalyses the breakdown of the diterpene (*E*,*E*)-geranyllinalool (C_{20}) or the sesquiterpene (*E*)-nerolidol (C_{15}) to the homoterpenes (*E*,*E*)-4,8,12-trimethyltrideca-

1,3,7,11-tetraene (TMTT, C_{16}) and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT, C_{11}), respectively (Lee et al. 2010). Just recently, Magnard et al. (2015) suggested that monoterpenoids—usually synthesized by the plastidic methylerythritol phosphate (MEP) pathway involving terpene synthases—can also be produced in the cytoplasm in cells of the petals of roses. In that case, the cytoplasmic Nudix hydrolase RhNUDX1 is involved in the synthesis of geraniol by converting geranyl diphosphate to geranyl monophosphate, the latter being the direct precursor of geraniol. Thus, this example represents a pathway for monoterpene synthesis independent of plastidic terpene synthases (Magnard et al. 2015). From these examples, we can conclude that the chemical diversity of floral scents can be explained by the number of different (alternative) biochemical pathways, the high number of enzymes and the multiple enzymatic modifications where VOCs or nonvolatile products of secondary metabolism are used as precursors.

11.3 Ecological Functions of Flower Scents

Flowers, as reproductive structures, are of special importance in the life cycles of plants. As most flowers provide resources such as nectar and pollen, they are also of special importance for obligate or facultative consumers of these resources. Many of these consumers are also pollinators, but to the detriment of the plants a lot of them are antagonistic agents that reduce the reproductive output of plants when they interact with flowers. Thus, plant fitness increases if flowers are adapted to both mutualists and antagonists. Floral scents possess attractive and defensive properties and thus are important traits for promoting or preventing interactions with various functional groups of flower visitors (Junker and Blüthgen 2010). For a full functionality of flowers, floral scents are usually complemented with other floral traits such as morphology and pigmentation (Junker and Parachnowitsch 2015). In the following, I will summarize the mechanisms that explain the multifunctionality of floral scents in interactions with pollinators, antagonistic animals and microorganisms. Additionally, I will emphasize that the dynamics of floral scent emissions—either following a diel rhythm or as a response to biotic interactions—allows flowers to quickly adapt their phenotype to current needs.

11.3.1 Attraction

Flower–pollinator interactions are either highly specialized where partners mutually depend on each other, are part of strongly generalized/opportunistic assemblages or are located somewhere along the continuum between specialization and generalization (Waser and Ollerton 2006). In systems where both partners mutually depend on each other, the plant and the pollinator often communicate via private channels, i.e. VOCs that are specific to this system and may only be perceived by the specialized pollinator (Raguso 2008b). A remarkable example of such a specialized system is the association between flowers that offer oil as a primary reward for pollinators and oil bees that specifically collect the oil from these flowers for nest construction and nutrition of their larvae. Although oil plant species belong to several families and oil bees belong to the Apidae or Melittidae, diacetin, a VOC that is likely synthesized by the same biochemical pathway as the oil reward, may be a universal signal that attracts the bees to the flowers (Schäffler et al. 2015). Interestingly, bee species not specialized on oil flowers do not seem to perceive diacetin (Schäffler et al. 2015). Thus, floral scent components such as diacetin have key functions in mediating highly specialized pollination systems, which are discussed in greater detail by Renee Borges (see Chap. 10). Likewise, flowers that rely on animals from the same taxonomical or functional group of pollinators have been shown to share floral scent characteristics (‘scent syndromes’, Dobson 2006). Bird–pollinated flowers are often weakly scented (Knudsen et al. 2004); in contrast, bats often find their floral resources based on sulfur-containing VOCs (Knudsen and Tollsten 1995). Flowers that are predominantly pollinated by butterflies (Andersson et al. 2002), moths (Knudsen and Tollsten 1993) or beetles (Jürgens et al. 2000) often share characteristics too (see Dobson 2006 for a comprehensive summary including further taxa such as flies and bees). Thus, in plant–pollinator interactions that are specialized to at least a certain degree, the positive valence of floral scents, i.e. the intrinsic behavioural quality (here positive attractiveness), represents a key feature in the maintenance of mutualistic interactions.

A large proportion of flowering plants is, however, visited and pollinated by more than one animal taxon or functional group (Ings et al. 2009; Gomez et al. 2015), which prevents an assignment of these flowers to a pollination or scent syndrome (Junker and Parachnowitsch 2015; Ollerton et al. 2009). Although in these systems the flowers often do not emit volatiles specifically evolved to address an animal taxon and the animals often do not possess a specifically adapted olfactory system, floral scents are important traits in maintaining flower constancy. Flower constancy is defined as the tendency of an animal individual to consecutively visit flowers of the same species ensuring pollen transfer between conspecific plants even in generalized systems, where animal species regularly visit the flowers of multiple plant species (Chittka and Thomson 2001). In the absence of signals specifically evolved to attract a main pollinator, mechanisms other than innate preferences are required. Since the pioneering work of Karl von Frisch (1967) on the highly generalized honeybee *Apis mellifera*, it has become well known that associative learning of flower volatiles and other floral stimuli is a cognitive process that helps animals to effectively exploit resources and also benefits plants due to conspecific pollen transfer. Although literature on associative learning is dominated by studies on honeybees, this ability is well known for a broad range of flower-visiting animals (e.g. Dupuy et al. 2006; Katzenberger et al. 2013). In a community study, it became obvious that prior experience of floral scents, a prerequisite for associative learning, strongly affected responses towards floral scent bouquets when tested in an olfactometer: animal species from various insect orders that had been caught from flowers that also served as scent sources in the olfactometer trials responded more positively to the scents than animals caught from flowers of other species (Junker et al. 2010b), demonstrating the general importance of associative learning in species-rich communities.

In principle, every neutral stimulus (conditioned stimulus) that may be irrelevant for an animal in the first place and not elicit an innate response can be associated with a stimulus (unconditioned stimulus) that elicits strong innate responses such as rewards (e.g. sugar) (Carlsson and Hansson 2006). After successful associative learning, animals reliably respond to conditioned stimuli that indicate the presence of rewards (Smith et al. 2006). As a restriction to the statement that ‘every’ stimulus can be a conditioned stimulus, it is worth noting that the ability to associate a stimulus with a reward depends on the animals being equipped with olfactory receptors that facilitate processing of the odours (Carlsson and Hansson 2006). In laboratory experiments, it has been shown that the salience of an odour (i.e. the perceptual quality by which an object stands out from its surrounding environment, independent of its valence) is positively correlated with the learning success of animals based on the same odour (Daly et al. 2007; Guerrieri et al. 2005; Katzenberger et al. 2013; Smith and Cobey 1994). Flowers that are pollinated by a broad taxonomical spectrum of animals may thus facilitate flower constancy by emitting bouquets comprising volatiles that are well perceived by a number of animal taxa, thereby increasing the salience of the flowers for the local pollinator assemblage and thereby potentially the pollination efficiency.

In conclusion, positive behaviour towards volatiles is a function of the valence as well as the salience of floral scents. The attractive function of scents in specialized systems may furthermore be reinforced if the positive valence of a compound is supported by a high salience of the same compound. This seems to be realized in the oil flower/oil bee system discussed above where diacetin has high values in the valence as well as in the salience. However, valence and salience are not always positively correlated, for example for *Helicoverpa armigera* moths (−)-α-pinene has a higher salience than (+)-α-pinene, but behavioral assays showed that the moths are more attracted to the latter (Hull et al. 2004) suggesting a higher valence value of (+)-α-pinene than (−)-α-pinene. The examples presented here suggest that valence and salience are key features explaining the attractiveness of floral scents in both specialized and generalized systems.

11.3.2 Repellence

The valence of floral scents is not restricted to attractiveness, but often includes aversion providing a defensive function to flowers. Terpenes, common constituents of floral scent bouquets, have long been known to have defensive properties mostly in the context of herbivory, whereas their function in floral ecology was long assumed to be restricted to the attraction of pollinators (Gershenzon and Dudareva 2007). This bias towards the attractive function of floral scents has been moderated in recent years with acknowledgement of the defensive functions of floral scents (Farré-Armengol et al. 2013; Muhlemann et al. 2014). Anton Kerner (1879) devoted a whole book on '*flowers and their unbidden guests*' to emphasizing the necessity of flowers to feature defensive means for keeping their '*unbidden guests*' at bay. Indeed, many animals represent floral antagonists that feed on floral tissues, steal rewards without pollination or use flowers as hunting sites and thereby reduce the visitation rates of pollinators (Dukas and Morse 2003; Hargreaves et al. 2009; Inouye 1980; McCall and Irwin 2006). Therefore, plant species have a selective advantage if they do not only attract pollinators and facilitate pollination but also adapt to floral antagonists by employing defensive features, such as morphological barriers (Galen 1999). In 1931, Robert Stäger (1931) accidentally made the first observation to indicate that volatiles may protect flowers against ants. During a field trip in the Alps, he forgot containers to sample ants and therefore asked his daughter to provide him with a container that previously contained her perfume. To Stäger's surprise, the ants did not survive as long as he expected from his earlier experience of keeping ants in containers and concluded that the perfume (containing floral scent compounds) was toxic to ants. In follow-up tests, he kept ants in containers with freshly picked flowers of several species and mostly made the same observation (Stäger 1931). The ant-repellent effect of floral scents has been confirmed several times in recent years (Galen et al. 2011; Junker and Blüthgen 2008; Junker et al. 2011c; Willmer et al. 2009). Likewise, florivorous crickets (Junker et al. 2010a) as well as spiders that potentially use flowers as sites to sit and wait for prey (Junker et al. 2011a) are repelled/deterred by common floral scent compounds. Interestingly, crab spiders adapted to flowers to prey on pollinators did not show any response to floral scents, neither positive nor negative (Junker et al. 2011a). The list of animals that are repelled by floral scents does not only include putative inefficient pollinators or antagonists but also animals that are well known for being important pollinators in many plant species. For instance, *Pieris rapae* butterflies are repelled by the scent of *Osmanthus fragrans* flowers (Omura et al. 2000) and the bumblebee *Bombus terrestris* as well as the honeybee *Apis mellifera* are repelled by scents emitted from *Achillea millefolium* flowers (Larue et al. 2016). In the latter cases, it remains unknown whether the repellent properties of the floral scents provide a selective advantage for the plants, e.g. due to the insects possibly being nectar and/or pollen thieves.

As mentioned earlier, floral scents in isolation are not the only traits that reduce or prevent visits by floral antagonists. Flowers often employ either morphological *or* chemical defences to protect themselves against detrimental visitors. For example, *Polemonium viscosum* flowers that are well protected against ant visits by a narrow nectar tube (morphological barrier) emit less 2-phenylethanol, an ant-repellent compound, than flowers of the same species with a wide nectar tube easily accessible to ants (Galen et al. 2011). The finding that flowers either possess morphological or chemical defences has been confirmed for large sets of flowering plant species (Junker et al. 2011b; Willmer et al. 2009), emphasizing the importance of floral defence in general. The attractive *and* repellent properties are thus of ecological importance and have the potential to explain a considerable amount of the interaction structure found in natural communities, where interactions between flowers and their visitors are non-randomly distributed (Junker et al. 2010b). The studies summarized above suggest that floral scent bouquets are clearly not only the result of selection by pollinators but also represent an adaptation to floral antagonists.

11.3.3 Regulation of Microbial Growth

Nectar is not only a valuable resource for mutualistic and antagonistic animals but also a source of carbon and amino acids for microorganisms such as yeast (Herrera et al. 2008) and bacteria (Alvarez-Perez et al. 2012; Fridman et al. 2012; Junker and Keller 2015). These microorganisms also colonize other flower organs such as petals, pollen, stamina and styles (Junker and Keller 2015; Fuernkranz et al. 2012; Junker et al. 2011d; Pozo et al. 2012). The yeast and bacteria communities associated with flowers usually differ in their composition from those found on other plant parts such as leaves or roots, suggesting that the microorganisms that are able to establish and proliferate on floral tissues are specifically adapted to this ephemeral habitat (Junker and Keller 2015; Junker and Tholl 2013). The establishment of bacteria on plant surfaces is controlled by a number of properties such as the cuticle properties, the availability of nutrients (Bodenhausen et al. 2014; Lindow and Brandl 2003), and clearly also by VOCs (recently reviewed by Junker and Tholl 2013). Floral VOCs have been shown to inhibit the growth of bacterial strains originating from leaves, while strains isolated from flowers and potentially adapted to these volatiles were less affected (Junker et al. 2011d). Huang et al. (2012) clearly demonstrated that (*E*)-β-caryophyllene predominantly emitted by the stigma of *Arabidopsis thaliana* flowers inhibits the growth of *Pseudomonas syringae* pv. *tomato* DC3000, a pathogen of Brassicaceae. VOCs emitted by plants do not only inhibit the growth of bacteria, but may also serve as carbon sources for other bacterial strains and thus have a growth promoting function for microbes, which has not been specifically shown for floral scents, but has been shown for secondary metabolites produced by roots (Del Giudice et al. 2008). These results indicate that floral scents, similar to the interactions with animals, may have both positive and negative effects on the establishment and growth of bacteria. Additionally, due to the omnipresence of bacteria and yeast, their role as floral pathogens (Buban et al. 2003), and their effects on pollinators (Junker et al. 2014; Herrera et al. 2013; Vannette et al. 2012), microorganisms, mutualistic and antagonistic animals, are likely to select for the composition of floral scent bouquets (Junker and Tholl 2013), which is a topic deserving of more experimental studies in the future.

11.3.4 Dynamics in Floral Scent Emission and Responses to Biotic Interactions

Like other traits featured by flowers, floral scents vary within and between individuals and populations (Soler et al. 2011; e.g. Knudsen 2002; Jhumur et al. 2008). However, unlike most other traits, floral scents can dynamically and quickly change over time even within individual flowers. Environmental conditions such as temperature may be a source of variation (Farré-Armengol et al. 2014). In addition, it has been shown that emission rates and scent composition follow a diel rhythm that matches the activity pattern of pollinators (Burdon et al. 2015; Dötterl et al. 2005) potentially to conserve resources at times when the receivers of attractive volatiles are inactive (Muhlemann et al. 2014). For instance, *Silene otites* flowers reach peak emission rates at night when pollinating moths and mosquitoes are active (Dötterl et al. 2012b), whereas flowers of *Lithophragma* species emit the largest amounts of volatiles when their day-active pollinating moths are in search of resources (Friberg et al. 2014). In *Salix caprea*, which is pollinated by bees during the day and by moths during the night, the composition of the scent bouquets changes in a diel rhythm according to the preferences of the pollinator that is active at a given time (Jürgens et al. 2014).

In addition to fixed diel rhythms of floral scent emission, flowers also flexibly respond to biotic interactions on flowers or other plant parts. Flowers that advertise their resources prior to pollination by attractive floral scents may reduce emission rates and thus attractiveness after pollination (Negre et al. 2003; Muhlemann et al. 2006). For the orchid *Ophrys sphegodes*, it has been described that pollinated flowers start to emit a non-attractive scent compound potentially to guide pollinators to non-pollinated flowers (Schiestl and Ayasse 2001). Likewise, flower damage by florivores has dramatic effects on the quantitative and qualitative emission of floral scents (Zangerl and Berenbaum 2009; Lucas-Barbosa et al. 2011). In addition to biotic interactions on flowers, interactions on leaves can also have effects on floral scent bouquets. For instance, male flowers of *Cucurbita pepo* subsp. *texana* alter their scent bouquets after artificial leaf damage predominantly by elevating the quantities of terpenoids (Theis et al. 2009). These herbivore-induced changes in floral scent bouquets may also affect flower visitor behaviour: Herbivore-induced defences in *Solanum peruvianum* led to the reduced performance of *Manduca sexta* caterpillars feeding on the leaves of this species; however, the visitation time and frequency of bumblebees were also reduced (Kessler and Halitschke 2009). The reduced attractiveness of flower scent of *S. peruvianum* to pollinators translated into reduced seed set of the flowers (Kessler et al. 2011). In contrast, despite marked differences in floral scent bouquets between plants that received herbivory and those with undamaged leaves in two *Brassica* species and associated changes in pollinator behaviour, the reproductive success of these plants remained unaffected (Bruinsma et al. 2014; Schiestl et al. 2014). A recent study by Lucas-Barbosa et al. (2015) demonstrated that the scent emitted by *Brassica nigra* flowers gives precise information about previous interactions with pollinators, herbivores and a combination of both. This variation in scent emission induced by biotic interactions has consequences for further interactions, such as with pollinators (Lucas-Barbosa et al. 2015). These studies demonstrate that intraspecific variation in flower scents is likely to be the result of whole-plant interactions of plant individuals (mediated by systemic plant responses to interactions), which affects interactions with pollinators. However, these studies also suggest that plant reproduction is often well buffered from these effects.

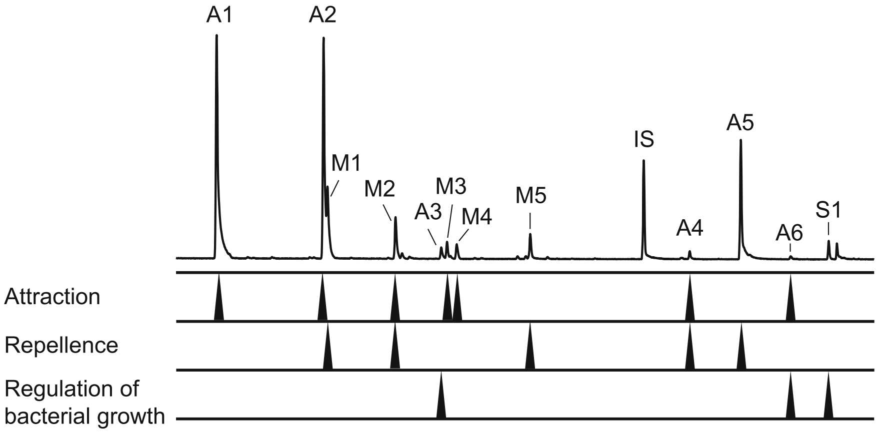
The dynamics described for the quantitative and qualitative emission rates of flower scents contrasts with the inflexibility of other flower traits, such as morphology, where changes in the phenotype relevant for pollinator behaviour usually occur much slower (but see Fründ et al. 2011). Thus, scents give flowers the flexibility to either synchronize with their pollinators in a diel rhythm or to quickly respond to their biotic environment and thus to potentially balance defence and attraction. Alternatively, herbivore-induced changes in floral scent bouquets may also represent a constraint with no consequences for plant reproduction. Thus, whether and under which circumstances biotically induced changes in floral scent bouquets are adaptive deserve further investigation.

11.4 The Right Mix for Multifunctional Scent Bouquets

The preceding paragraphs illustrated the multifunctional character of floral scent bouquets. Floral scents mediate interactions with mutualists and antagonists, arthropods and vertebrates, eukaryotes and prokaryotes and thereby increase the reproductive success of plants. However, studies on the ecological functions of floral scents usually focus on a single interaction partner, e.g. the main pollinator of the focal plant species. Such studies often present a single volatile or a relatively small number of components that elicit a response, such as pollinator attraction (Riffell et al. 2009; Schäffler et al. 2015) or repellence of an antagonist (Junker et al. 2011c), in isolation. The behavioural relevance of single (or few) compounds in flower–pollinator interactions seems to be the rule rather than an exception and has been shown in many case studies. For instance, *Hoplitis adunca* bees specialized on genera from the family Boraginaceae are innately attracted to 1,4-benzoquinone, which is emitted by the host plants of this bee species (Burger et al. 2012). Experienced bees may, however, use additional compounds for host finding (Burger et al. 2012). Dötterl and Vereecken (2010) compiled a list of floral scent compounds that have been described to elicit positive responses in bee species. Multiple other examples show that individual (or few) compounds within bouquets are suited to mediating interactions in specialized pollinator systems (e.g. Chen et al. 2009; Dötterl et al. 2006; Svensson et al. 2010). As a consequence, the presence of a single compound in one species—but not in another species with an otherwise similar scent bouquet—may be sufficient for reproductive isolation (Waelti et al. 2008). In more generalized flower–pollinator interactions, where associative learning plays a major role, the importance of ‘key compounds’ is also evident. In proboscis extension reflex experiments, it became evident that honeybees that are trained to associate odour blends with rewards use only a small subset of the compounds to positively respond to the blends (Reinhard et al. 2010). These key compounds are characterized by the ability of the animals to learn them individually, their reliability to inform about rewards, or their salience (Daly et al. 2007; Katzenberger et al. 2013; Laloi et al. 2000; Wright and Smith 2004). Compared to the many examples showing that one or two key substances are required for the maintenance of interactions, relatively few examples show that several compounds (in specific ratios) need to be presented for the full behavioural response. For instance, flowers of *Datura wrightii* emit at least 60 compounds, nine of which are required to attract the pollinating moth *Manduca sexta* (Riffell et al. 2009). Besides mutualistic interactions, antagonistic interactions are also mediated by individual compounds that are either used by antagonists to locate the flowers (Andrews et al. 2007), utilized by the flowers to repel the antagonists (Junker and Blüthgen 2008; Junker et al. 2011c; Kessler and Baldwin 2007; Kessler et al. 2008) or used to inhibit the growth of pathogenic bacteria (Huang et al. 2012).

These results may suggest that only one or a few from the many volatiles present in a floral scent bouquet are ecologically relevant and, thus, adaptive. Indeed some volatiles emitted by flowers may result from biochemical constraints such as components produced from multi-product enzymes that may synthesize the functional (adaptive) compound but also other non-adaptive VOCs. Many terpene synthases, for example, are capable of converting a single precursor to multiple mono- or sesquiterpenes (Degenhardt et al. 2009). However, such biochemical constraints alone may not be sufficient to explain the high number of volatiles identified in many floral scent bouquets. It is thus likely that those compounds that are not required for the interaction considered in a given study have other functions in interactions with organisms that have not been investigated in the same study. In nature, the pair-wise interactions mediated by individual scent compounds do not occur in isolation but are embedded in complex communities consisting of other plant species and of further animals and microorganisms that are in search of floral resources. Accordingly, the qualitative and quantitative composition of floral scent bouquets may be shaped by multiple interaction partners (Junker and Blüthgen 2010; Junker and Tholl 2013; Kessler et al. 2008; Raguso 2008b), potentially including other plant species (Pierik et al. 2014), which suggests that the presence of most of the volatiles may be adaptive.

Figure 11.2 visualizes the concept of multifunctional scent bouquets by compiling results from several studies that demonstrated responses of diverse organisms to individual compounds that are part of the floral scent bouquet of *Phlox paniculata* (Polemoniaceae, Junker et al. 2011c). In this hypothetical example, each of the scent compounds has one or more functions including attraction, repellence and the regulation of bacterial growth, thus allowing the plant to mediate interactions in diverse communities. Few studies have directly tested the effects of floral scent bouquets on multiple organisms, but those that did support the concept of the multifunctional scent bouquet. For example, phenylacetaldehyde, benzaldehyde and p-anisaldehyde, which are emitted by *Cirsium arvense* flowers, were shown



Compound		Function	Interaction partner	Reference
Aromatics				
A1	Benzaldehyde	Attraction	Bee (*Apis mellifera*)	(Theis 2006)
A2	Phenylacetaldehyde	Attraction	Ant (Formicidae)	(Theis 2006)
		Attraction	Bee (*Apis mellifera*)	(Theis 2006)
		Attraction	Moth (*Pseudoplusia includens*)	(Meagher 2002)
A3	Phenylacetonitrile	Growth inhibition	Bacteria (diverse bacteria)	(Junker et al. 2011d)
A4	Eugenol	Attraction	Bumblebee (*Bombus terrestris*)	(Junker and Blüthgen 2010a)
		Attraction	Thrips (*Thrips flavus*)	(Kirk 1985)
		Repellence	Ant (*Lasius niger*)	(Junker and Blüthgen 2010a)
A5	Methyleugenol	Repellence	Ant (*Linepithema humile*)	(Shorey et al. 1996)
A6	Methylisoeugenol	Attraction	Fly (*Psila rosae*)	(Guerin et al. 1983)
		Growth inhibition	Bacteria (*Campylobacter jejuni*)	(Rossi et al. 2007)
Monoterpenoids				
M1	Ocimene	Repellence	Moth (*Manduca sexta*)	(Kessler and Baldwin 2007)
M2	Linalool	Antifeedant	Bush cricket (*Metrioptera bicolor*)	(Junker et al. 2010a)
		Attraction	Bee (*Apis mellifera*)	(Theis 2006)
		Repellence	Ant (*Camponotus floridanus*)	(Junker and Blüthgen 2008)
		Repellence	Ant (*Lasius fuliginosus*)	(Junker and Blüthgen 2008)
		Repellence	Ant (*Lasius niger*)	(Junker et al. 2011c)
		Repellence	Ant (*Monomorium floricola*)	(Junker and Blüthgen 2008)
		Repellence	Fly (*Musca domestica*)	(Lee et al. 2003)
M3 / 4	Lilac aldehydes	Attraction	Moth (*Hadena bicruris*)	(Dötterl et al. 2006)
M5	Lilac alcohol	Repellence	Hoverflies (*Episyrphus balteatus*)	(Boachon et al. 2015)
		Repellence	Thrips (*Frankliniella occidentalis*)	(Boachon et al. 2015)
Sesquiterpenoids				
S1	Germacrene D	Growth promotion	Bacteria (diverse bacteria)	(Del Giudice et al. 2008)*

Fig. 11.2 Multifunctional floral scent bouquet. Different functions are assigned to each of the volatiles found in the floral scent bouquet of *Phlox paniculata* (Polemoniaceae, Junker et al. 2011c) that mediate the interactions in a hypothetical flower visitor community. Note that these functions were not revealed in the context of the ecology of *P. paniculata* but were extracted from studies dealing with other plant species or investigated for the volatiles in isolation. Nonetheless, this compilation emphasizes the potential multifunctional character of floral scent bouquets, which has so far not been examined in a single species with consideration of such a diversity of potential interaction partners. *Asterisk* the study by Del Giudice et al. (2008) did not directly test the effect of germacrene D on bacteria, but indicates that bacteria are capable of using many sesquiterpenes as a carbon source. *IS* internal standard used to quantify the compounds emitted by *P. paniculata*. Figure modified from Junker (2010)

to attract pollinators, whereas benzaldehyde and linalool attract and repel florivores, respectively (Theis 2006). Kessler and Baldwin (2007) individually tested the responses of moths, hummingbirds and ants to scent compounds found in *Nicotiana attenuata* flowers and demonstrated that each of the taxa displayed different behaviours towards the volatiles. Using the same plant species, the authors also demonstrated that both repellent and attractive compounds are required for maximal reproductive success (Kessler et al. 2008). Diel rhythms in floral scents, where emission rates of compounds that either attract day or night active pollinators, are synchronized with the activity pattern of the pollinators (e.g. Jürgens et al. 2014, see above for further examples), are also in accordance with the multifunctionality of scent bouquets. In a community approach, Junker et al. (2010b) showed that diverse insect species differentially respond to the same floral scent bouquets, i.e. bouquets attractive to some insect species were repellent to others that alternatively visited flowers of other plant species.

Many studies have thus indicated that most pair-wise interactions between flowers and other organisms are mediated by a small subset of the volatiles emitted by flowers, often by a single key compound. However, the multiple and diverse potential interaction partners encountered by flowers in natural ecosystems may have selected for scent bouquets comprising diverse volatiles, enabling the flower to specifically select mutualistic partners and to avoid or reduce interactions with antagonistic agents.

11.5 Integrating the Chemical Ecology of Flowers into Community Ecological Research

As outlined above, multifunctional scent bouquets are traits that enable flowering plant species to mediate interactions with diverse organisms. Thus, floral scents increase the reproductive outcome of plants if interactions with mutualists are promoted while interactions with antagonists are synchronously reduced or prevented. Plant species are characterized by unique floral scent bouquets (Knudsen and Gershenzon 2006), which means that each plant species contributes to the pool of volatiles emitted by a community. Thus, each species may add novel functions and new types of interactions with other organisms to plant communities. Most studies on the ecology of flower scent, however, either involve a narrow taxonomical spectrum of plants (mostly one species and often variation in a genus or family) or a narrow taxonomical spectrum of animals (flowers pollinated by the same animal species, genus or family). These restrictions limit knowledge on the effects of flower scent at the community ecology scale. In general, VOCs emitted by plants have mostly been neglected in community-wide assessments of functional traits, potentially because specialized equipment and/or expertise in both chemical and community ecology are needed. A recent study by Filella et al. (2013) allows first insights into the community-wide emission rates of flowers; floral emission rates of terpenoids across all plant species within a community were heterogeneously distributed across the flowering season. Species that flowered early in the season were more strongly scented than species that flowered later in the season. This seasonal variation in emission rates was negatively correlated to relative abundance of pollinators (i.e. number of pollinators per flower) suggesting that plants that compete with a high number of co-flowering species for pollinators invest more resources in flower advertisement using VOCs than species that flower at times when intra- and interspecific competition is relaxed (Filella et al. 2013). In the same study, β-ocimene was identified as the main attractant in plant species flowering early in the season in low abundances, which may allow these species to compete with plant species that flowered at the same time but in higher abundances. Because the study by Filella et al. (2013) focuses exclusively on flower terpenoid emissions, no information on the overall diversity of flower scent compounds is available. However, studies that investigated VOCs emitted by vegetative plant parts of multiple species within communities suggest an enormous diversity in emission rates and in the identity of scent compounds (Owen et al. 2001; Courtois et al. 2009). A study on the volatiles emitted by the bark of South American tropical tree species impressively demonstrated the great diversity of volatile compounds by showing that the vast majority of VOCs identified were found in just one or a few of the 55 species sampled (Courtois et al. 2009). Likewise, the number of floral scent compounds strongly increased with the number of plant species sampled, both in communities in the Hawai'i Volcanoes National Park (Kuppler and Junker, unpublished) and in the Austrian Alps (Larue-Kontic and Junker, unpublished, Fig. 11.3). The volatile accumulation curves presented in Fig. 11.3 are far from saturation, indicating that each plant species features a new set of VOCs, which is also supported by the high number of VOCs that exclusively occur in a single plant species, and thus are species-specific (inset in Fig. 11.3).

The functional diversity is a non-redundant component of overall diversity of communities (Devictor et al. 2010) and describes the characteristics, distribution and relative abundance of traits within communities (Cadotte et al. 2013). Functionally, diverse communities are characterized by species that feature a high interspecific variation in traits. Thus, the species or phylogenetic diversity of a plant community is not necessarily positively correlated to the functional diversity of the same community (Junker et al. 2015). For instance, the volatile accumulation curves presented in Fig. 11.3 indicate that both the Hawaiian as well as the Austrian plant communities are functionally hyperdiverse in floral scent emissions because each species adds novel compounds (and potentially novel functions) to the community. The investigation of the functional diversity of plant communities promotes the understanding of how functional traits are linked to environmental gradients, niche theory, structuring of interactions, community assembly and ecosystem functioning (Cadotte et al. 2013; Whitham et al. 2006; Junker et al. 2015; Mouchet et al. 2010). For instance, functionally diverse plant communities feature increased primary productivity (Liu et al. 2015; Mason and de Bello 2013; Turnbull et al. 2013), are more stable (Turnbull et al. 2013), are less susceptible to invasions (Funk et al. 2008) and support a higher diversity of species at higher trophic levels

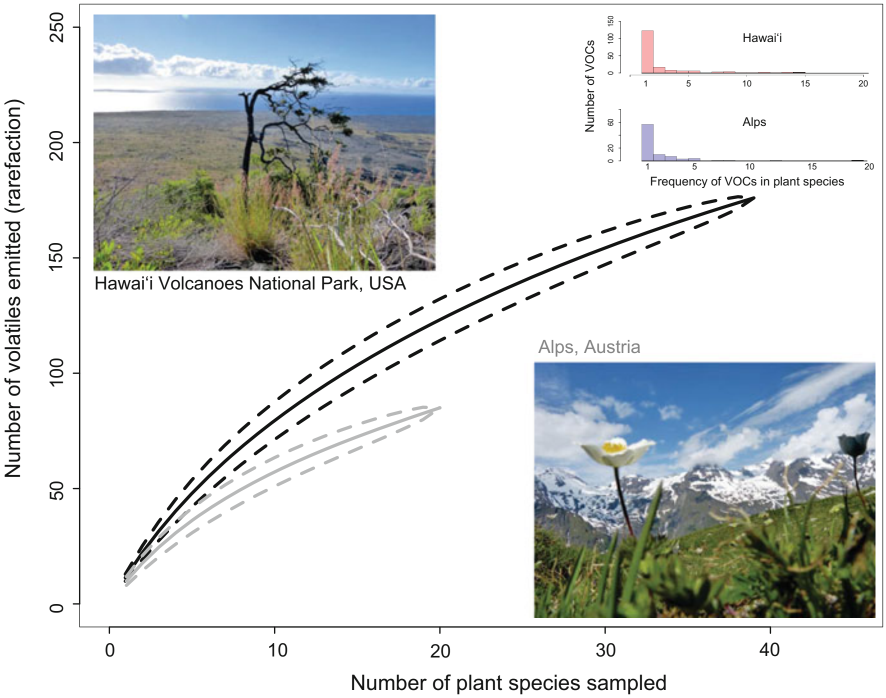


Fig. 11.3 Richness of VOCs emitted from flowers within species rich communities in Hawai'i ($n = 33$ communities) and in the Austrian Alps ($n = 2$ communities). Volatile accumulation curves (rarefaction, mean ± SD) show the number of volatiles as a function of the number of plant species sampled. The inset (*upper right corner*) shows the number of VOCs that have been found in a given number of plant species. Sources: Hawai'i (Kuppler and Junker, unpublished), Alps (Larue-Kontic and Junker, unpublished). Photographs by RRJ

(Junker et al. 2015). These ecosystem functions are affected by either multiple or specific traits, but not all functions are mediated by the same traits (Cadotte et al. 2013; Spasojevic and Suding 2012; Kraft et al. 2015). In the context of floral ecology, it has been shown that the diversity of one trophic level is often associated with the number of species in another trophic level, which often has been attributed to the functional diversity of both plants and animals (Fontaine et al. 2006; Biesmeijer et al. 2006; Campbell et al. 2012; Fründ et al. 2013). A key to understanding the mechanisms that underlie these correlated diversities of primary producers and their consumers is linking niche theory with community ecology (McGill et al. 2006), which supports the view that the presence of functional groups (e.g. the presence or absence of volatiles with specific functions for species interactions) rather than species predicts ecosystem processes (Junker et al. 2015). For example, the finding that pollinators partition floral resources based on the match between their proboscis length and the depth of the flowers' corolla, meaning that a functional group of plants (e.g. those with long corollas) supports a functional group of pollinators (e.g. those with long proboscis') (Graham and Jones 1996; Johnson 1986), has recently been applied to explain community-wide interaction patterns between diverse flowering plant species and their flower visitors (Junker et al. 2013; Stang et al. 2007). These examples and those that also consider flower traits apart from morphology such as colour and rewards (Carvalheiro et al. 2014; Junker et al. 2013; Kaiser-Bunbury et al. 2014) also support the notion that the functional diversity of flower traits predicts the number and identity of flower visitors that are sustained by the plants (Junker et al. 2015). Despite the crucial role of the functional diversity of plant communities for ecosystem functioning, species interactions and thus overall biodiversity (Cadotte et al. 2013; McGill et al. 2006) as well as the importance of floral scents in mediating interactions with multiple organisms, empirical studies linking chemical ecology of floral scents with community ecological approaches are virtually absent (Raguso 2012). However, the functional hyperdiversity of floral scent compounds encountered within communities (Fig. 11.3) suggests a significant impact of this trait on interaction structure (Junker et al. 2010b) and ecosystem properties (Randlkofer et al. 2010).

Recent studies suggest that these findings can also be applied to the functional diversity of floral scents. Flower–visitor interactions are usually more complementarily specialized than many other trophic interactions, meaning that species interact with a specific subset of partners that are potentially available, which results in non-random interactions between flowers and animals (Blüthgen et al. 2007). The deviation from a random distribution of insects on flowers, i.e. more or less interactions than expected by chance, was correlated to positive or negative responses of the insects to flower scents (Junker et al. 2010b). Thus, behavioural responses to olfactory cues explained a considerable part of the interaction structure in a natural species-rich community (Junker et al. 2010b). The pronounced role of floral scents for foraging decisions of flower visitors was also supported in a study combining experimental modifications of flower scents and field observations. After the reciprocal application of floral scent extracts of one species to the flowers of another species, the interaction structure strongly changed both qualitatively as well as quantitatively (Larue et al. 2016). Flower visitors that had been observed on only one of the plant species frequently visited the other plant species after application of the extracts, whereas others had reduced interaction frequency upon application of the extract of the other species (Larue et al. 2016). Olfactometer trials confirmed that attraction and repellence were responsible for these effects. The restructuring of the interaction networks resulted in more similar (qualitatively and quantitatively) visitor assemblages associated with the plant species and thus in a more generalized network pattern (Larue et al. 2016). These findings clearly demonstrate the pronounced role of floral scents in resource or pollinator partitioning among animal and plant species, facilitating co-existence of species and thus contributing to the maintenance of diverse communities. The examples provided here allow a first glimpse at the significance of floral scent bouquets on community ecological processes. Future studies are, however, clearly needed to investigate and compare the functional diversity of floral scents across communities, ecosystems and biomes and to evaluate the relative importance of floral scents and other flower traits in community composition and other ecosystem processes.

11.6 Conclusion

Floral scents are a remarkable feature of angiosperms characterized by their hyperdiversity, multifunctionality, flexibility and their range of action. These characteristics allow flowers to mediate interactions with pro- and eukaryotic mutualists and antagonists and to quickly adapt their phenotype to prevailing environmental conditions and biotic factors. Furthermore, albeit not covered in this chapter, the potential of floral scents to travel large distances in the field (Riffell et al. 2014; Ackerman 1986) increases the spatial radius of flowers to communicate with interaction partners. Thus, floral scents have crucial functions in the reproductive biology of angiosperms, which often cannot be accomplished by other flower traits such as morphology, colouration or rewards. Accordingly, as also pointed out by Raguso (2008a), it is essential to integrate the chemical ecology of flowers into pollination ecology in order to comprehensively understand the complex interplay between plants and their biotic environment. Beyond that, I made an attempt to promote the consideration of floral scents in community ecological approaches, where VOCs should be treated as other (often morphological) functional traits that regulate species interactions and other important ecosystem processes. Currently, we have limited understanding of the relative importance of floral scents in community processes. However, the few available studies combining the chemical ecology of floral scents with community ecological approaches, along with the numerous studies demonstrating the multifunctionality and flexibility of floral scent emissions, give rise to the assumption that floral scents are indeed of major importance. Another emerging field, which is also related to community ecology, but at a much smaller scale, is floral microbial ecology. Likewise, the first information concerning the interference in floral ecology by microorganisms and the roles of VOCs in regulating—either positively or negatively—the growth of microorganisms is very promising. I hope that this chapter will further stimulate research that considers whole communities of diverse plant, animal and microbial species that will complement the valuable studies on pair-wise interactions mediated by floral scents. Such studies are needed to fully appreciate the fascinating multifunctionality of floral scents.

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Part III
Mechanisms of Volatile Detection by Plants

Chapter 12
Plant Electrophysiology: Early Stages of the Plant Response to Chemical Signals

Simon A. Zebelo and Massimo E. Maffei

Abstract Plant defence strategies start at the plant cell plasma membrane, where volatile organic compounds (VOCs) induced by insect herbivores or plant pathogens interact chemically and trigger plant signalling molecules. The earliest plant responses for the perception of VOCs are ion flux imbalances generated in the plant cell plasma membrane at the perception zone. This different charge distribution generates variation in the plasma transmembrane potential (V_m), which is the first event preceding the regulation of signal transduction pathways and gene expression. Change in the V_m can be through either an increase (hyperpolarization) or a decrease (depolarization) in the membrane potential. Here, we review recent advances in electrophysiological methods for the study of the early events of VOC perception and the correlation between V_m depolarization and plant signal transduction pathways leading to changes in gene expression.

12.1 Introduction

The plasma membrane represents the sensing element that recognizes changes in the cell-surrounding environment and starts cascades of electric signalling, eventually resulting in specific plant responses. Leaf damage, infection by plant pathogens, and feeding by insect herbivores induce the delivery of elicitors or the generation of plant cell wall-derived elicitors that may bind to specific receptors in the plant plasma membrane. Emerging evidence indicates that many high-affinity receptors for insect herbivores (Maffei et al. 2007a, 2012), plant pathogens (Elmore

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and Coaker 2011), and allelochemicals (Roshchina 2001) are located in the plant cell plasma membrane. The elicitor–receptor interaction results in variation in the plasma transmembrane potential (V_m), which is defined as the difference in the electrochemical gradient between the interior and exterior of the plant cell. This variation can lead to either more positive (depolarization) or more negative (hyperpolarization) V_m values, and these events eventually lead to the generation of signalling cascades.

Intercellular plasma membrane depolarization was recorded in *Nitella* sp. cells for the first time in 1930, earlier than the first intracellular electrical signal recording in animal cells (Nastuk and Hodgkin 1950; Tasaki 1952). Recently, most of the chemistry of the neuromotoric system of animals has been found in plants; for example, neurotransmitters such as acetylcholine and cellular messengers and cellular motors such as calmodulin and actin (Cao et al. 2006). Although this nerve-like cellular machinery never develops the same degree of complexity as in animal nerves, a simple plant neural network is formed, especially within phloem cells, which is responsible for the symplastic plasmodesmata-mediated communication over long distances (Fromm and Lautner 2007; Bricchi et al. 2013). Despite the direct effect of herbivore or microbial elicitors, plant membranes can also respond to VOCs either produced by the same plant or emitted by neighboring plants. Here, we summarize recent research on V_m variation as a common event of plant–VOC interactions. We will start this overview by describing the techniques currently used in plant electrophysiology to detect V_m variation.

12.2 Electrophysiological Methods for the Evaluation of V_m Variations

The system we currently use to measure V_m in leaf segments is the result of many technical tests, which eventually gave a useful set of electrical, electronic, and hydraulic instruments for conducting on-line (or real-time) recording of electrical variation in plant plasma transmembrane potential. This system was initially developed to measure membrane potential variations upon the effect of the phytoalexin isosakuranetin (5,7-dihydroxy 4′-methoxy flavanone) on potassium uptake in wheat root segments (Sacco and Maffei 1997) and the allelopathic effects of *Mentha* x *piperita* essential oil and monoterpenes on cucumber root membrane potential (Maffei et al. 2001). The system consists mainly of a homemade block constructed from Plexiglas, a polymethyl methacrylate (PMMA) polymer, or Teflon, a more inert polymer of polytetrafluoroethylene (PTFE) and some wells and sockets as shown in Fig. 12.1. We use this block to perfuse specific chemicals (including plant volatiles) dissolved in a buffer through a leaf segment, which allows electrophysiological measurements to be made from living tissues. A small square section of a leaf is incubated in a fresh buffer and then placed in the central socket of the block, and the V_m is measured as described in Fig. 12.1. A peristaltic pump—normally operating at a speed of 1 ml min^{-1}—pumps the buffer

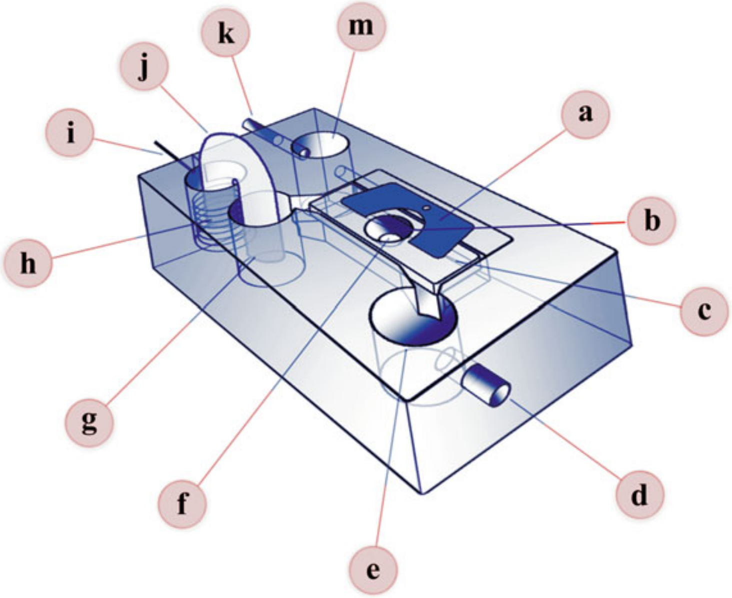

Fig. 12.1 Schematic representation of the system used for the evaluation of V_m in leaf segments. Modified from (Maffei and Bossi 2006). (**a**) Stainless steel holed blade that holds down the top and allows the probe to reach the leaf fragment and directly measure V_m; (**b**) hole giving access to the probe; (**c**) plastic lid with hole that fixes the leaf fragment for V_m detection; (**d**) excess buffered solution is extracted from the central socket through this exit with a vacuum pump; (**e**) well used to remove excess buffer; (**f**) central socket where a small square part of a leaf is placed; (**g**) well containing one of the two ends of the salt bridge; (**h**) well containing one of the two ends of the salt bridge and the silver wired solenoid; (**i**) wire solenoid connected to the ground; (**j**) salt bridge providing the electric link between the ground and the buffer solution; it is a curled glass pipe filled with agarose containing 3 M KCl; (**k**) inlet for buffer flowing from the peristaltic pump; (**m**) well where bubbles, if present, can easily emerge and dissolve

through the system. The core of the equipment is the electrical circuit, which allows V_m measurement. In order to measure V_m, we use very fine tipped (2–3 μm) borosilicate glass capillaries (WPI Inc, model 1B150F-4), which are made with a capillary puller (Nareshige model PE-21) and filled with a 3 M KCl solution diluted with ultrapure water (Millipore) (Maffei et al. 2004). Due to the very fine tip, the 3 M KCl solution in the inner part of the glass electrode permits an efficient electrical conductance with a very low (fM) loss of ions from the electrode to the cellular matrix. Glass microelectrodes are directly connected to a probe (WPI Inc.) by means of an electrode holder (WPI Inc.); this probe does the first step of cleaning and stabilizing the signal and is connected to a signal amplifier (WPI Inc.; e.g., model Electro 705). The amplifier takes the electrical signal coming from the cell and brings it amplified, cleaned, and stabilized to an oscilloscope (e.g., Tektronics model TDS 210), for further digital elaboration and data storage. The signal is measured and recorded in mV. The oscilloscope also allows visualizing the wave of the electrical signal, the shape of which gives important information about cell condition, electrode integrity, and, in general, the electrical conditions of the entire system. The oscilloscope is also plugged to the ground, to complete the electrical circuit; ground is represented by the silver wire solenoid in the well. The ground is a special silver–silver chloride electrode, which acts as a signal transducer by converting ionic currents in a solution to an electric current within a wire, the same operation done by the probe attached to the glass electrode.

All instruments and electrical devices are very sensitive to both physical vibration and environmental electrical noises; thus all equipment is mounted on a stable work table, electrically grounded, and kept in a Faraday cage; all cables and wires, when not shielded properly, are wrapped with aluminum foil in order to reduce noise. Obtaining a good V_m is a very delicate operation, and the use of a micromanipulator, which can move in three directions, is fundamental. A stereo microscope or a special video camera is also needed in order to see exactly where to position the electrode onto the leaf tissue. Data recording is performed on a normal paper recorder or directly to a computer equipped with data logger software.

V_m can also be recorded in intact plant leaves. Therefore, we developed a chamber where molecules diffused in air can be focussed on leaves. Figure 12.2 shows how a plant cutting bearing entire leaves can be analyzed. The membrane potential is captured with a glass electrode that is positioned directly on a leaf that is

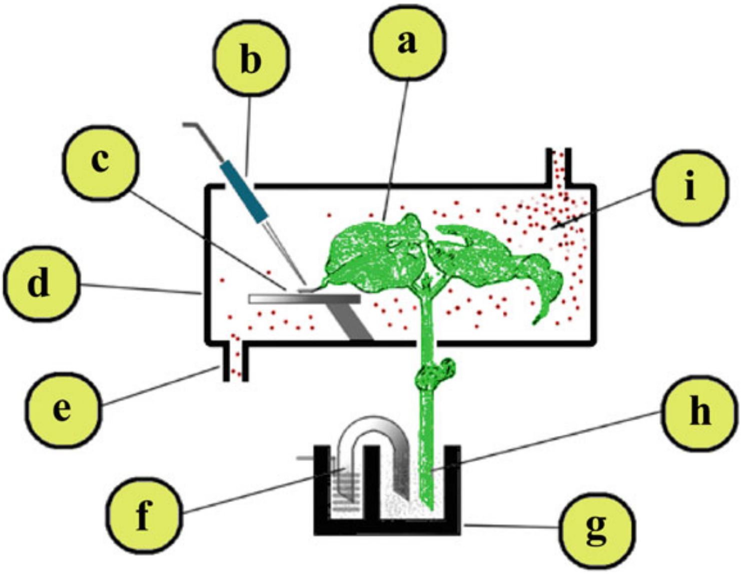

Fig. 12.2 Schematic representation of the system used for the detection of V_m in intact leaves of plant cuttings. Modified from (Maffei and Bossi 2006). (**a**) Leaves perceive and respond to volatile organic compounds that saturate the chamber; (**b**) V_m is detected with a probe; (**c**) holder; (**d**) Teflon chamber; (**e**) outlet for excess volatiles; (**f**) salt bridge (see also Fig. 12.1 for details); (**g**) Teflon box hosting the stem and the salt bridge; (**h**) stem immersed in a buffered solution; (**i**) inlet for volatile organic compounds

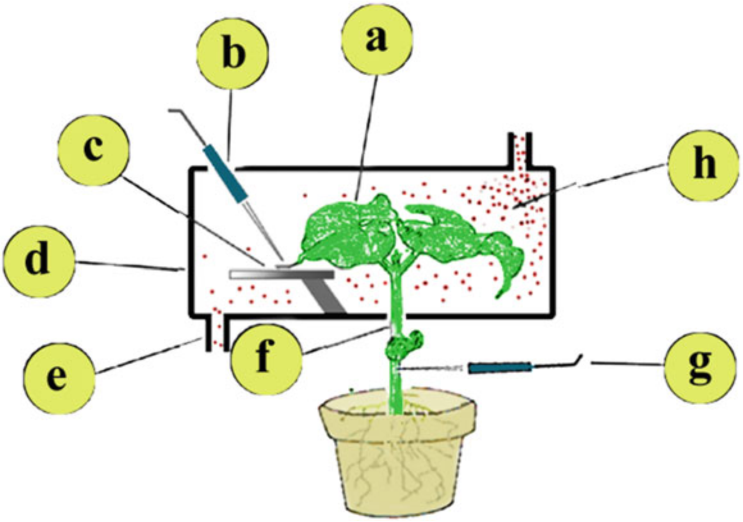

Fig. 12.3 Scheme of the system used for the detection of V_m in intact leaves of potted plants. Modified from (Maffei and Bossi 2006). (**a**) Leaves perceive and respond to volatile organic compounds that saturate the chamber; (b) V_m is detected with a probe; (**c**) holder; (**d**) Teflon chamber; (**e**) outlet for excess volatiles; (**f**) potted plant; (**g**) ground electrode inserted in the stem; (**h**) inlet for volatile organic compounds

fixed on a holder. The V_m of intact plant tissue is measured with two glass micropipettes filled with 3 M KCl and with a tip resistance of 4–10 MΩ. With the help of a micromanipulator, the first electrode is inserted into the mesophyll cells of the leaf. The electrode is then connected to a probe and to the amplifier. The position of the second electrode depends on the experimental setup. When the experiment utilizes a potted plant, the electrode is inserted into the plant stem phloem cells using a micromanipulator and is then connected to the ground port (Fig. 12.3) (Zebelo et al. 2012). When the experiment is with a plant cutting (Fig. 12.2), the plant cutting's stem is immersed in an ionic solution in a Teflon well. A salt bridge allows the plant to be in contact with the ground through a special coiled silver–silver chloride electrode, which acts as a signal transducer by converting ionic currents in the solution to an electric current within a wire connected to the ground port of the oscilloscope (Maffei and Bossi 2006).

12.3 Mechanisms of Electrical Signalling

Now that we have shown how to detect V_m variation in plants, we will turn our attention to the known electrical signalling mechanisms that plants adopt to respond to insect and pathogen attacks, and in the perception of VOCs. In principle, the systemic signalling induced by biotic stressors and volatile signals is transduced by either chemical or electrical signals (Heil and Silva Bueno 2007; Maffei et al. 2007b; Mithöfer et al. 2009; Zebelo and Maffei 2012b; Zimmermann et al. 2009). Three broad mechanisms are recognized for the transmission of electrical signals, those involving action potentials (APs), variation potentials (VPs), and system potentials (SPs).

Action Potentials In higher plants, action potentials (APs) are defined generically as a long distance signalling system that may act to potentiate a host response to subsequent signals delivered through alternative long distance information packages. An AP is a momentary change in electrical potential of plant cells in response to environmental stimuli that eventually leads to intercellular and intracellular communication. A number of substances strongly depolarize the plasma membrane and thus presumably activate (voltage-gated) ion channels. Excitation of APs in plant cells depends on Ca^{2+}, Cl^{-}, and K^{+} ions (Zebelo and Maffei 2012a; Zimmermann et al. 2009; Felle and Zimmermann 2007). Felle and Zimmermann (2007) showed that although in principle it is possible that (anion) channels are directly activated by depolarization, the temporal sequence of the ion flux kinetics of barley leaves shows that Ca^{2+} is lost from the apoplast well before apoplastic anion concentration (measured as Cl^{-}) starts to increase. Therefore, channel activity is involved in APs, and the more the channels are activated the more rapid the depolarization will be, eventually leading to an accelerated depolarization that is measured as membrane potential "break-through," typical of an AP. When plasma membrane depolarization reaches a certain critical threshold level, AP is generated according to the "all or none" rule (Pyatygin et al. 2008). Like herbivores (and their oral secretions) and plant pathogens (and their elicitors), volatile signalling is known to cause APs, which usually appear as a single pulse; in rare cases, several repeated pulses are generated during VOC perception, and then APs propagate along the conducting bundles beyond the area of its generation. APs generated by VOCs propagate as fast electrical signals that travel through the entire plant from the point of VOC interception (Zebelo et al. 2012). APs generated by herbivory propagate at a speed up to 40 m s^{-1} (Volkov and Mwesigwa 2000). Using the aphid technique, Fromm and Bauer (1994) measured electric and cold-shock triggered APs in maize sieve tubes travelling at 3–5 cm s^{-1}. In the presence of leaf-feeding larvae of the Colorado potato beetle (*Leptinotarsa decemlineata*), the speed at which insect-induced APs moved downward through the stem was about 0.05 cm s^{-1} (Fig. 12.4). Zebelo and coworkers demonstrated that herbivore-induced plant volatiles (HIPVs) trigger APs and VPs on nearby receiver plants (Zebelo et al. 2012).

Since APs are released by agents added in a realistic concentration range, it is suggested that they may serve as the first and fast systemic signals following attack from pathogens (Felle and Zimmermann 2007). Indeed, APs with a complete Ca^{2+} signature are required for activation of plant defence responses against pathogens (Blume et al. 2000). Similar Ca^{2+} signatures were recorded in tomato plants exposed to herbivore-induced plant volatiles and synthetic VOCs (Zebelo

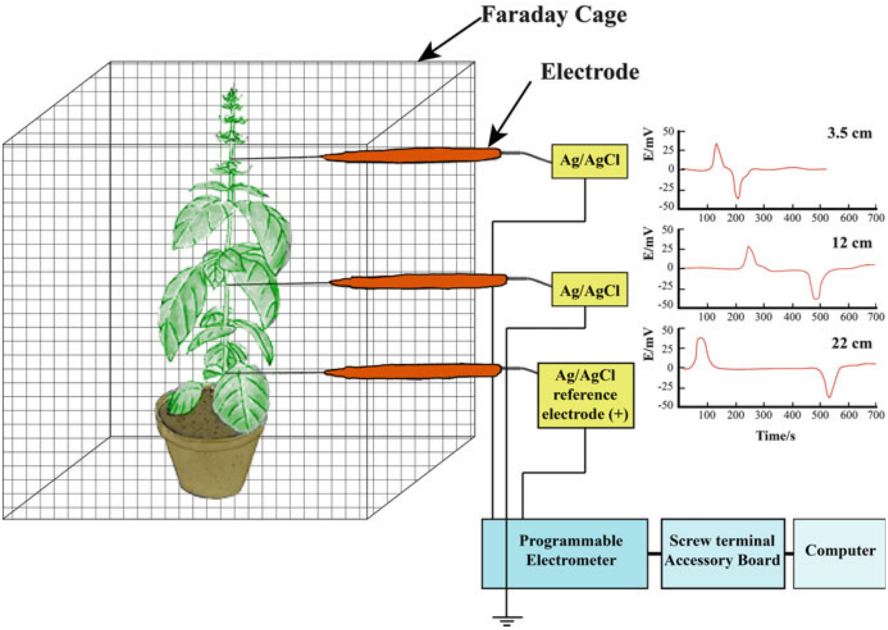

Fig. 12.4 Detection of action potentials in plants. All bioelectrochemical measurements are conducted at room temperature inside a Faraday cage. Ag/AgCl electrodes are connected to a programmable electrometer-amplifier with a high input impedance (>200 TΩ) through a triaxial cable. A computer with a multi-input–output plugin data acquisition board is interfaced with the electrometer and used to record the digital data. On the *right side* is an example of APs from measurements of plants challenged by the Colorado potato beetle on young terminal leaves. Distances between electrodes are indicated. Adapted from Volkov and Haack (1995)

et al. 2012). Figure 12.4 shows a typical configuration of apparatus for measurement of APs upon herbivory.

Variation Potentials Variation potentials (VPs) are defined as slow oscillations of the plasma transmembrane potential. In plants, VPs are characterized by amplitudes and speeds that decrease with increasing distance from the site injured by insect herbivores (Maffei and Bossi 2006) or plant pathogen infection (Elmore and Coaker 2011; Bricchi et al. 2012). While APs do not carry much information about the nature or intensity of the damage caused by the insect herbivore or plant pathogen infection, VPs are modulated in amplitude as well as in their interdependent ion fluxes, from which the plant or the affected organ may be able to gain information about the nature and intensity of the biotic injury (Zebelo and Maffei 2012a). V_m changes upon biotic stress have been associated with other important signalling events such as intracellular Ca^{2+} concentration variations (see below) and oxidative stress caused by either H_2O_2 or NO (Maffei et al. 2004; Maischak et al. 2007; Zebelo and Maffei 2012a; Bricchi et al. 2010). Mechanically damaged Lima bean leaves react fast and dramatically to H_2O_2 by undergoing a strong V_m

depolarization. However, leaves wounded by the herbivore *Spodoptera littoralis* already show a reduced starting V_m with the consequence of a dramatically lower or even no responsiveness to H_2O_2 application (Maffei et al. 2006). Thus, the depolarization of the V_m by the action of herbivory is linked to a reduction in downstream responses of attacked leaves to signalling molecules such as H_2O_2 and NO (Bricchi et al. 2010).

Manipulation of VPs by pathogens might result in significant lowering of host tissue responses and activation of defence. For instance, in *Arabidopsis thaliana*, significant VP variations were recorded after sixteen hours of *Pseudomonas syringae* DC3000 infection. At the same time, a large number of defence genes were downregulated including the respiratory burst oxidase protein C (RbohC)/ NADPH oxidase (At5g51060), NADP-dependent oxidoreductase (At5g16960), universal stress protein (USP) family protein (At3g62550), mechanosensitive ion channel domain-containing protein (At1g53470), and a MATE efflux protein (At1g58340) (Bricchi et al. 2012).

Herbivorous insects and their oral secretions, plant pathogens and their elicitors, as well as volatile signalling are known to cause both APs and VPs, but it is still unclear whether herbivorous insects and volatile signalling can cause SPs (Bricchi et al. 2013; Maffei et al. 2007b; Zebelo and Maffei 2012b). SPs have recently been described as novel electrical long distance apoplastic signals in plants induced by wounding and acting as a forerunner of slower travelling chemical signals (Zebelo and Maffei 2012a; Zimmermann et al. 2009). SPs serve as a backup to APs and VPs and overlap with APs and VPs in some instances. Having this brief background about SPs, it is hard to exclude the occurrence of SPs during volatile signalling (Zebelo and Maffei 2012a).

12.4 Generation of Electrical Signals Upon Biotic Stress

12.4.1 The Plant Plasma Transmembrane Potential (V_m) Responds to Biotic Stress

Plant defence strategies against biotic stressors are generally orchestrated as a network of perception systems, which start at the front line of damage (the plasma membrane) where insect herbivores or plant pathogens interact with plants physically (by mechanical damage) and chemically (by introducing elicitors or triggering plant-derived signalling molecules). There a fast state change (the V_m variation) triggers gene expression-independent reactions, eventually leading to the activation of signalling pathways able to affect the cell, its surroundings, and the whole plant, with concerted local and systemic responses. In contrast to hydraulic, mechanical, and hormonal signal transduction, electrical signals are able to deliver fast information over long distances. To better understand the role of V_m variation as a triggering event, we will address some key questions on (i) how the plasma transmembrane potential (V_m) responds to biotic stress and (ii) how ion channels and transporters are involved in the perturbation of the chemiosmotic balance.

As stated above, the cell plasma membrane is the only cellular component with direct contact to the environment and represents the sensing element able to recognize changes and to initiate cascades of events eventually leading to specific responses. Changes in V_m or modulation of ion fluxes at the plasma membrane level are among the fastest cellular responses to biotic and abiotic stresses (Shabala and Bose 2012; Maffei et al. 2007a). The candidate ion species responsible for V_m variations in plant cells upon biotic stress are calcium (Ca^{+2}), protons (H^+), potassium (K^+), and chlorine (Cl^{-1}).

Herbivory-Dependent V_m *Variations* Herbivory-dependent V_m variations result from the combination of two concomitant processes: (1) the mechanical damage of the plant tissues and (2) the introduction of oral secretion (OS) of the feeding insect into wounded tissues. Thus, the attacked plant faces both a mechanical and a chemical challenge (Bricchi et al. 2010; Mithöfer et al. 2009). Application of insect OS to a mechanical injury can mimic most plant responses to herbivory (Reymond et al. 2004), suggesting that OS elicitors are major contributing factors to a plant recognizing insect attacks (Bricchi et al. 2012; Consales et al. 2012; Maffei et al. 2012). Indeed, several elicitors have been isolated from insect OS that trigger plant defences against herbivory, such as β-glucosidase (Mattiacci et al. 1995), volicitin (a fatty acid–amino acid conjugate) (Halitschke et al. 2001), caeliferins (Alborn et al. 2007), inceptins (Schmelz et al. 2006), and a still uncharacterized polysaccharide elicitor (Bricchi et al. 2013). Lepidopteran OS consists of mandibular and other labial secretions, glandular secretions from the ventral eversible gland, and regurgitate from the digestive tract (Felton 2008; Zebelo and Maffei 2012b). In the *S. littoralis–Phaseolus lunatus* interaction, both direct herbivory and the insect's oral secretions have been demonstrated to induce a fast plant cell V_m depolarization (Bricchi et al. 2010, 2012; Maffei and Bossi 2006; Maffei et al. 2004), and the same response was shown when *S. littoralis* was feeding on other higher plant species such as *A. thaliana* (Zebelo and Maffei 2012b; Bricchi et al. 2013) and *Ginkgo biloba* (Mohanta et al. 2012) and lower plant species such as the fern *Pteris vittata* (Imbiscuso et al. 2009). Interestingly, a significant V_m depolarization was observed at almost every stylet puncture during phloem feeding by *Myzus persicae* (Bricchi et al. 2012).

During insect-elicited V_m variations, the plant cell responds with a V_m depolarization, the duration of which depends on the nature of the biotic attack. For instance, the V_m depolarization is much more rapid in response to feeding by *S. littoralis* (30 min) than by the aphid *Myzus persicae* (4–6 h) (Bricchi et al. 2012). Irrespective of the nature of the biotroph, this event occurs at the same intensity, which in Arabidopsis corresponds to a V_m depolarization of about 40 mV. The different timing of V_m depolarization appears to be associated with the mode of biotic damage. The fast clipping and consistent plant tissue removal by chewing herbivores evidently induces a "quantitative" response that is proportional to the amount of tissue damage; however, stylet probing and phloem feeding by aphids induces less damage, which requires more time for a plant response. This implies that the speed of the V_m response allows setting a time scale for conducting comparative genome-wide analyses. In fact, in Arabidopsis, a clear relationship between V_m depolarization and gene expression was found. When genome analyses were conducted at the point of V_m depolarization, feeding by the aphid *M. persicae* regulated a wider array of Arabidopsis genes than feeding by *S. littoralis* (Bricchi et al. 2012).

Pathogen-Dependent V_m *Variations* In plant–pathogen interactions, V_m depolarization is a reliable early indicator of the leaf hypersensitive response (HR) (Pike et al. 2005). *P. syringae* DC3000 infection is also able to induce a V_m depolarization in Arabidopsis with the same magnitude of V_m depolarization as recorded for aphid and herbivore attacks, suggesting the presence of a V_m threshold that has to be reached for a successful herbivory/infection response (Bricchi et al. 2012; Pike et al. 2005). Bacterial flagellin represents one of the best studied pathogen-associated molecular patterns (PAMP) that induce V_m depolarization in mesophyll cells and in root hair cells of Arabidopsis (Jeworutzki et al. 2010). Elicitors isolated from the fungus *Cladosporium fulvum* were also demonstrated to induce V_m depolarization in tomato plants (Gelli et al. 1997). Plasma membrane ion channels are rapidly activated by pathogen infection or by elicitor treatment of plant cells (Fisahn et al. 2004). However, bacterial growth and tissue damage take time, which appears to be proportional to the timing of V_m depolarization (14–16 h) (Bricchi et al. 2012). At the time of V_m depolarization, an almost completely opposite state of regulation was observed for Arabidopsis damaged by the aphid *M. persicae* and the pathogen *P. syringae* DC3000, with the former suppressing and the latter activating defence responses (Bricchi et al. 2012). In parsley cells treated with Pep-13, an oligopeptide fragment of a 42-kDa *Phytophthora sojae* cell wall glycoprotein, extracellular alkalinization, Ca^{2+} influx, and efflux of K^+ and Cl^- lead to V_m depolarization (Fisahn et al. 2004; Blume et al. 2000).

Abiotic and biotic stresses, including mechanical wounding and insect attack, elicit signals that trigger a phosphorylation cascade leading to jasmonic acid (JA) biosynthesis. JA is a key regulatory component in defence-signalling pathways. The JA level increases in corn (*Zea mays*) and hybrid poplar (*Populus deltoides* × *nigra*) seedlings after exposure to green leaf volatiles (Engelberth et al. 2004).

12.4.2 V_m Changes and Ion Variations in Response to Biotic Stress

Plant ion channels are transmembrane proteins located in the plant cell membranes that mediate ion fluxes across the cell compartments (Wang 2012). The hydrophilic pore structure of plant ion channels allows the passage of ions through the membranes at extremely high rates (106–108 ions per second through one channel protein) driven by transmembrane electrochemical potentials (Maathuis et al. 1997). Different plant ion channels are selectively expressed in specific tissues or cells, where they perform the appointed functions to facilitate the physiological processes of those tissues or cells (Wang 2012). Recall that V_m variations are caused by an unbalanced ion distribution across the plasma membrane and that depolarization occurs when cations (such as K^+ and Ca^{2+}) are allowed to enter the cell or anions (e.g., Cl^-) are allowed to exit the cell. Furthermore, hyperpolarization depends mainly on the activity of the plasma membrane H^+-ATPase or when inward anion channels or outward cation channels are opened (Maffei et al. 2004).

Herbivory-Dependent Ion Fluctuations and Their Effects on the V_m An electrophysiological approach called the planar (black) lipid bilayer technique (BLM) is widely used to elucidate the molecular mechanisms and activities of various biologically active substances (Winterhalter 2000). Using BLM, Boland and coworkers demonstrated that insect-derived elicitors (e.g., fatty-acid–glutamine conjugates, LeaGln) and the *Spodoptera exigua*-derived OS directly interact with artificial lipid bilayers to generate channel-like activities that are highly conductive and selective for certain ions (Maischak et al. 2007).

In general, upon herbivory, the increase in cytosolic calcium precedes membrane depolarization (Maffei et al. 2007a). Ca^{2+} is one of the principal intracellular messengers and during electric signal generation and propagation, Ca^{2+} enters the cytoplasm through voltage-gated Ca^{2+} channels located at the plasma membrane and other membranes of intracellular stores (Arimura and Maffei 2010). Herbivore feeding causes a dramatic Ca^{2+} cytosolic ion influx limited to a few cell layers lining the wounded zone (Zebelo and Maffei 2015). This response is limited to herbivory or biotrophic activity, since neither single nor repeated mechanical wounding events are able to induce significant changes in cytosolic Ca^{2+} ion influx (Bricchi et al. 2010). Insect feeding and isolated insect-derived elicitors are known to cause activation of Ca^{2+} channels (Arimura and Maffei 2010; Maffei et al. 2007a), and these events have been associated with V_m depolarization (Maffei et al. 2004).

However, upon herbivory, the Ca^{2+} signalling depends on the symplastic continuity granted by functioning plasmodesmata. We have recently made the surprising observation that an Arabidopsis line (*pdko3*) mutated in genes encoding plasmodesmal proteins is defective in some of the typical plant responses to herbivory. Following herbivory and the release of OS, both the *pdko3* and wild-type (WT) plants showed increased accumulation of cytosolic Ca^{2+}, but, unlike WT plants, the mutant line showed an almost complete loss of V_m depolarization. Thus, the mutations in genes for plasmodesmal proteins have provided valuable genetic tools for the dissection of the complex spectrum of responses to herbivory and shown us that the responses to herbivory imply a Ca^{2+}-independent V_m depolarization (Bricchi et al. 2013).

Increased K^+ channel activity was observed when Arabidopsis plants were damaged by *S. littoralis* feeding, or OS was introduced to mechanically damaged leaves (Bricchi et al. 2013). Potassium represents the major osmotically active cation in plant cells and is fundamental for plant functions such as control of membrane potential (Geiger et al. 2009; Lebaudy et al. 2007). Upon herbivory, the increased concentration of cytosolic Ca^{2+} triggers the opening of inward rectifier K^+ channels that are the major factor responsible for herbivore-induced V_m depolarization. In the *pdko3* Arabidopsis mutant, it appears that the connection between increased calcium and K^+ channel activation is broken leading to significantly decreased V_m depolarization, although lower absolute levels of cytosolic calcium in *pdko3* leaves opens the possibility of a threshold effect on K^+ channel activation (Bricchi et al. 2013).

Microbe-Induced Variation in Ion Homeostasis and the Effect on V_m Receptor-mediated recruitment of calcium stores and activation of plasma membrane anion channels represent initial steps in pathogen recognition and innate immunity-related signalling (Elmore and Coaker 2011). The increased cytoplasmic Ca^{2+} concentration observed in response to stress induced by bacteria and fungi involves extracellular polysaccharides, peptidoglycans, lipo-oligosaccharides, or chitin and appears to be an essential, common event after recognition of microbes by plant cells (Aslam et al. 2009; Lecourieux et al. 2005; Miya et al. 2007; Nurnberger and Scheel 2001; Romeis et al. 2001).

The use of ion-selective electrodes and imaging of simultaneous changes in cytosolic-free Ca^{2+} concentration allowed identification of the ion species involved in membrane depolarization following bacterial flagellin (flg22) treatment (Jeworutzki et al. 2010). Application of 10 nM flg22 to Arabidopsis leaves and roots induced a strong V_m depolarization after a lag phase of 1–3 min, with the magnitude and velocity of the depolarization dependent on the concentration at which flg22 was applied. Aequorin is a calcium-activated photoprotein; its apoprotein is called apoaequorin, and its prosthetic group is called the luciferin. Stimulation of Arabidopsis plants constitutively expressing apoaequorin to monitor the cytosolic Ca^{2+} concentration after application of flg22 resulted in a dose-dependent transient rise in cytosolic Ca^{2+}. When membrane potential recordings and Ca^{2+} imaging experiments were performed simultaneously, flg22 addition was found to cause a V_m depolarization and a cytosolic Ca^{2+} increase, which occurred within the same time frame (Jeworutzki et al. 2010).

Cryptogein is a 10-kD protein secreted by the oomycete *Phytophthora cryptogea* that induces a hypersensitive response in tobacco (*Nicotiana tabacum*) plants and systemic acquired resistance against various pathogens. Within the first 5 min after infection, cryptogein induces an anion efflux and a Ca^{+2} influx, which gives rise to a fast and large V_m depolarization. The latter, in cryptogenin-treated plants, has been associated with the inhibition of glucose uptake, which is symported with H^+ and, thus, depends on the transmembrane electrochemical potential difference (Bourque et al. 2002). Elicitors from *C. fulvum* activate tomato plasma membrane Ca^{2+} permeable channels resulting in increased cytosolic Ca^{2+} concentrations (Gelli et al. 1997).

Alamethicin is an elicitor from the fungus *Trichoderma viride* and is well known to produce pores in artificial membranes as well as in animal and plant cell membranes (Duclohier et al. 2003). Alamethicin induces pore formation in the tonoplast membrane of the plant *Chara corallina*, and these pores are conductive depending on the polarity of the voltage applied in the plasma membranes (Luhring et al. 2007).

Unlike other plant–pathogen interactions and in a similar fashion to plant responses to herbivory, Ca^{2+} signalling appears to be nonessential to the recognition of the early stages of viral infection. Shabala et al. (2010) observed significant changes in K^+ fluxes as early as 10 min after viral inoculation, which were mediated by depolarization-activated outward-rectifying K^+ channels. This may suggest that viral infections trigger a different mechanism of plant defence signalling compared to signals derived from other microbial pathogens; hence, altered Ca^{2+} fluxes across the plasma membrane may not be a common prerequisite for all elicitor-activated defence reactions.

VOC-Dependent Ion Fluctuations and Their Effects on V_m Compared to herbivore- and microbe-dependent ion fluctuations, there is limited information on the role of VOC-dependent ion fluctuations and their effects on the V_m. Zebelo and coworkers showed that VOCs emitted by tomato plants damaged by the herbivore *Spodoptera littoralis* trigger a significant V_m depolarization in the mesophyll cells of receiver tomato plants (Zebelo et al. 2012). In the same study, VOCs from mechanically damaged and control unwounded tomato plants did not exert significant differences in the V_m of receiver tomato plants compared to the effects of clean air. In a real-time experiment using confocal laser scanning microscopy (CLSM), tomato plants treated with the fluorescent calcium indicator Calcium orange were exposed to herbivore-induced plant VOCs and showed an increase in cytosolic calcium concentration $[Ca^{2+}]_{cyt}$ (Zebelo et al. 2012). These results show that plant perception of volatile cues from the surrounding environment is mediated by early events, occurring within seconds and involving the alteration of the plasma membrane potential and increases in the cytosolic calcium. GLVs are produced as long as herbivory is present, therefore the continuous emission of these molecules signals to neighbor plants the persistency of herbivore attack. Although the volatile language is still difficult to decipher, we found that low molecular weight VOCs such as GLVs prompt a faster and stronger V_m and calcium response when compared to higher molecular weight compounds such as monoterpenes and sesquiterpenes, which appear to act only on the V_m component of the plant cell response (Zebelo et al. 2012).

12.4.3 Effects of Biotic Stress on Transporter Activity

Transporters are specialized proteins that assist in the movement of small molecules, ions, macromolecules, peptides, and lipids across a biological membrane.

The plasma membrane H^+-ATPases are the primary pumps responsible for the establishment of cellular membrane potential in plants (Boller and Felix 2009). Plasma membrane H^+-ATPases use energy derived from ATP hydrolysis to pump protons to the extracellular space and inside the vacuole, thus creating and maintaining a negative V_m potential (Elmore and Coaker 2011). Therefore, any alteration in H^+-ATPase activity leads to V_m variation, with a depolarizing effect caused by the reduction of its ability to expel protons from the cytosol. In addition to regulating basic aspects of plant cell function, these enzymes contribute to signalling events in response to diverse environmental stimuli. H^+-ATPases are dynamically regulated during plant immune responses, and quantitative proteomics studies have demonstrated complex spatial and temporal modulation of plasma membrane H^+-ATPase activity during early pathogen recognition (Keinath et al. 2010; Nuhse et al. 2007) and insect herbivore recognition (Bricchi et al. 2013; Schaller and Oecking 1999). For instance, in response to pathogens, an increase in H^+-ATPase activity (i.e., V_m hyperpolarization) has been demonstrated in tomato (Veraestrella et al. 1994) and barley (Knogge 1996), whereas the inhibition of the H^+-ATPase (i.e., V_m depolarization) is exploited by pathogens in order to overcome plant resistance (Zhou et al. 2000). The pea pathogen, *Mycosphaerella pinodes*, inhibits H^+-ATPase activity in the pea plasma membrane through the action of the suppressor of phytoalexin accumulation, supprescin B (Kato et al. 1993).

H^+-ATPases and Ca^{2+}-ATPases are members of a class known as P-type ATPases. In response to insect herbivory, Ca^{2+} ions are released into the cytosol via channel proteins and pumped back into their stores (organelles) and the apoplast via Ca^{2+}-ATPase pumps (Bricchi et al. 2010; Maffei et al. 2004). Ca^{2+}-ATPases are regulated by herbivore OS (Maischak et al. 2007). Given the numerous roles of P-ATPase activity in plant cell physiology, it is no wonder that pathogens have evolved mechanisms to target these enzymes (Elmore and Coaker 2011).

Fusicoccin is produced by the fungal pathogen *Fusicoccum* (*Phomopsis*) *amygdale* and functions by locking the interaction between 14-3-3 proteins and the C-terminal regulatory domain of the plasma membrane H^+-ATPase, which leads to constitutive activation of this hydrogen pump and causes a constant V_m hyperpolarization (Baunsgaard et al. 1998). The mechanism for the reverse effect, where some still unknown biotic elicitors may interfere with the activation of the H^+-ATPase rendering the pump unable to repolarize a depolarized V_m, is particularly intriguing.

12.5 Concluding Remarks

Depolarization of the V_m is one of the first responses to biotic stress of the plasma membrane, which mainly depends on ion fluxes, including the opening of K^+ channels and the release of calcium from internal stores or through influx from the apoplast. These early events are followed by the activation of signalling pathways, eventually leading to plant responses to biotic stress (Fig. 12.5).

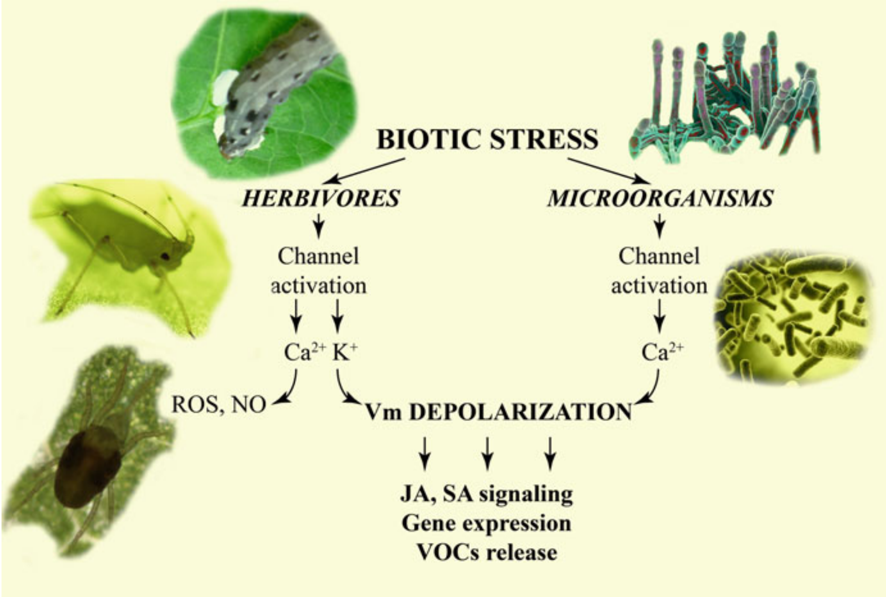

Fig. 12.5 V_m depolarization is a common event upon biotic stress. Insect herbivores induce the opening of both calcium and potassium channels. Potassium channel activity is the main factor responsible for V_m depolarization, whereas increased cytosolic calcium activates ROS and NO (Bricchi et al. 2013). Microorganisms activate calcium channels inducing plant cell V_m depolarization through the concerted action of anion and cation channels. V_m depolarization is then followed by activation of jasmonic acid (JA) and salicylic acid (SA) signalling pathways eventually leading to plant responses to biotic stress, including gene expression and the release of herbivore-induced and microbe-induced volatile organic compounds (VOC)

Thus, electrophysiology is indeed a valuable tool to study and understand early events in plant interactions with other organisms (including other plants), and V_m evaluation, more than the single patch analysis, gives a tissue image of cooperative interplay among wounded and unwounded cells.

Understanding fast responses of plants to the surrounding environment is of interest not only from an ecological and evolutionary perspective but also for the development of novel crop protection strategies. Owing to the massive damage that herbivores and pathogens cause to valuable crops, the deciphering of early signals from plants represents one of the most exciting fields of research in the first line of defence.

Three areas where future efforts might result in major breakthroughs are related to the identification of herbivore-specific signal molecules, their recognition, and further signal transduction. The challenge for further research is to determine their mode of action, whether these signals are transduced by receptor-mediated processes or simply interact with the plant membranes and thereby initiate signal transduction pathways. One approach to achieve this goal might be the use of plant mutants that are not responsive to a particular herbivory-related signal;

indeed, the Arabidopsis *pdko3* mutant was instrumental to dissecting early and late events in plant responses to herbivory (Bricchi et al. 2013). Characterization and the use of such mutants could result in the identification of genes encoding proteins involved in signal perception. Not only can such studies uncover individual signalling pathways, but they can also establish links in a network of alternative routes regulating the multitude of inducible plant defences. Much more has to be done in this field, but the promising results obtained in intact rooted plants following biotic and abiotic stress may lead to interesting new discoveries.

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Chapter 13
Uptake and Conversion of Volatile Compounds in Plant–Plant Communication

Koichi Sugimoto, Kenji Matsui, and Junji Takabayashi

Abstract Volatile organic compounds emitted from plants have an important role in communication between plants and other organisms (e.g. plant–pollinator, plant–herbivore and plant–carnivore communication). Recent studies have revealed a novel mechanism of volatile-mediated plant–plant communication. Here, plants take up volatiles through the stomata and by adsorption on the leaf surface. The volatiles are then processed within leaf tissues. Reduction and esterification of compounds increase their volatility, and the converted volatiles are emitted again into the air. Volatiles taken up by a plant also undergo glycosylation and glutathionylation, resulting in their conversion to non-volatile compounds that have ecological functions. For example, one of the glycosylated compounds, (*Z*)-3-hexenyl vicianoside, functions in plant defences against insect herbivory. Conversion to non-volatile forms would enable uninjured plants to be more defended against herbivores moving from neighbouring herbivore-infested plants. Uptake and conversion of volatile compounds in plants is discussed in this chapter.

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13.1 Introduction

13.1.1 Detection of Environmental Changes by Plants

Plants grow under fluctuating environmental conditions. Their phenological responses indicate that they are able to detect long-term environmental changes. For example, plants have their own specific flowering seasons (Aikawa et al. 2010). Plants are also affected by short-term changes caused by abiotic factors such as temperature, humidity and light intensity (Loughrin et al. 1994; Takabayashi et al. 1994; Maeda et al. 2001). Additionally, plants can detect possible biotic stresses such as herbivory and/or pathogen infestation by using volatiles. For example, herbivore-damaged plants emit a mixture of volatile compounds that are detected by neighbouring plants. The volatiles from herbivore-infested plants act as a signal to trigger defensive responses against herbivores in the neighbouring plants (Yoneya and Takabayashi 2014). We refer to this process as volatile-mediated plant–plant communication. In this chapter, we provide a brief overview of the ecological functions of plant volatiles, and then discuss recent progress regarding the mechanisms that plants use to detect volatiles, with particular focus on volatile uptake and processing.

13.1.2 Ecological Functions of Plant Volatiles

Plant volatiles may provide information regarding plant condition. For example, volatiles from fresh leaves, fruits and vegetables indicate that the plant material may be edible. Similarly, volatiles released from older or decaying plant tissues indicate that the material may be inedible. Likewise, herbivores may use plant volatiles to distinguish between suitable and unsuitable food. Uninfested leaves emit a specific mixture of volatiles that can be detected by herbivores to identify the presence of food. Foraging adult herbivores also use volatiles from infested plants to determine appropriate/inappropriate hosts, i.e. different volatiles function as attractants or repellents (e.g. Horiuchi et al. 2003; Yoneya et al. 2010). Plant volatiles can influence the behaviour of organisms other than herbivores. For example, flower and fruit volatiles can affect the behaviour of pollinators and seed dispersers (Rodríguez et al. 2013; Valenta et al. 2013; de Vega et al. 2014), and volatiles from plants infested by herbivorous arthropods attract carnivorous natural enemies of herbivores (for reviews: Takabayashi and Dicke 1996; Pare and Tumlinson 1999; Arimura et al. 2009; Dicke 2009; Reddy 2012; Takabayashi 2014).

When a volatile compound mediates interactions between plants and other organisms, it is classified as an infochemical, a chemical that, in the natural context, conveys information in an interaction between two individuals, evoking a behavioural or physiological response in the receiver (Dicke and Sabelis 1988). In plant–animal interactions, plants have long been considered to be emitters of volatile infochemicals; however, a number of studies published since 2000 have demonstrated that plants also have the ability to respond to volatile infochemicals emitted from neighbouring plants. Therefore, plants also serve as infochemical receivers (Yoneya and Takabayashi 2014).

13.1.3 Specificity of Plant–Plant Communication Mediated by Volatiles

Plant responses to volatiles are affected by the chemical structure, composition and abundance of the volatile compounds. For example, in response to volatiles from artificially damaged conspecifics, young *Chrysanthemum cinerariaefolium* seedlings increased the production of pyrethrins. Artificially damaged leaves emitted (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*Z*)-3-hexenyl acetate and (*E*)-β-farnesene as dominant wound-induced volatiles. Exposing uninjured seedlings to a mixture of these authentic volatiles at concentrations mimicking those of volatiles emitted from wounded seedlings resulted in increased pyrethrin levels in the uninjured seedlings (Kikuta et al. 2011). In this case, a mixture of all five compounds was necessary to induce pyrethrin production. Moreover, exposed *C. cinerariaefolium* seedlings respond to the mixture only within a narrow concentration range for each constituent, and the highest response was observed when the volatile concentrations were similar to those of volatiles emitted from injured plants (Kikuta et al. 2011).

Another example of plant responses to volatiles involves *Arabidopsis thaliana* and synthetic green leaf volatiles (Kishimoto et al. 2005). Treatment of *A. thaliana* with *allo*-ocimene (monoterpene), (*E*)-2-hexenal, (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate (green leaf volatiles) resulted in a chemical species-specific induction of defensive genes. *A. thaliana* plants also had different morphological responses in the roots when exposed to different enantiomers of borneol or bornyl acetate (Horiuchi et al. 2007). The enantioselective responses imply stereospecific recognition of volatile compounds in plants.

13.2 Route of Volatile Uptake

Regarding plant volatile perception, it is important to consider the routes through which airborne volatile organic compounds are transported into tissues. The general route of gas exchange in plants is through the stomata, which transport H_2O, CO_2 and O_2 to and from the mesophyll cells for photosynthesis. There is evidence indicating that volatile organic compounds are incorporated into plants through the stomata (Tani and Hewitt 2009; Tani et al. 2010). When *Quercus* species were exposed to methacrolein or methyl vinyl ketone under light conditions, there was a correlation between the volatile uptake rate by plants and their stomatal conductance (Tani et al. 2010), suggesting that the volatiles were incorporated into leaf tissues through the stomata (Fig. 13.1, route 1).

Choh et al. (2004) reported adsorption of volatile organic compounds on the leaf surface of a plant. They showed that volatiles emitted from lima bean leaves infested by two-spotted spider mites (*Tetranychus urticae*) were adsorbed on the surface of neighbouring uninfested lima bean leaves. Some of the adsorbed volatile compounds

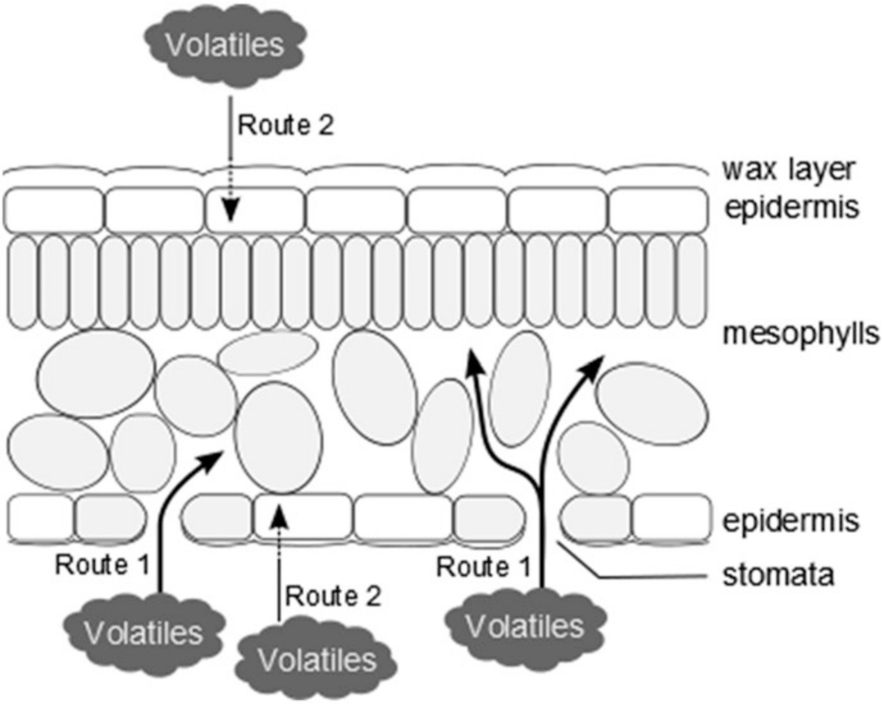

Fig. 13.1 Deduced routes of volatile uptake in leaf tissue. Some volatiles enter the intercellular space through stomata (route 1). This has been confirmed by measuring uptake rates when stomata are open and closed. Other volatiles are known to be absorbed and taken up through the leaf surface (route 2)

are re-emitted in the air (Choh et al. 2004), but some may be transferred into plant tissues. This possibility is supported by Tani and Hewitt (2009); even in a darkened enclosed space, peace lily plants (*Spathiphyllum clevelandii*) continued to take up volatile ketones from the air, suggesting that the lily plants incorporated ketone compounds by a route other than the stomata. It is plausible that the volatile ketones enter the plants by direct infiltration through the epidermis (Fig. 13.1, route 2).

13.3 Processing of Volatiles in Plants

13.3.1 Volatiles of Precursors of Phytohormones

The next step is the processing of the incorporated volatiles in plants. The processing of volatile phytohormones or volatile precursors of phytohormones is one example of what occurs to volatiles once they are incorporated by plants. Exogenously applied methyl jasmonate is hydrolysed by esterases to yield free jasmonic acid (Wu et al. 2008). Because endogenous jasmonic acid is converted into an actual signalling molecule, jasmonoyl-L-isoleucine, through the original biosynthetic pathway (Staswick and Tiryaki 2004; Svyatyna et al. 2014; Matsuura et al. 2012), exogenously obtained jasmonic acid would also be converted into jasmonoyl-L-isoleucine. Similarly, methyl salicylate is hydrolysed to generate the active form of salicylic acid (Forouhar et al. 2005), which induces defence responses against pathogens (Kumar and Klessig 2003). When *ent*-kaurene, a volatile precursor of gibberellins, was emitted from neighbouring Arabidopsis mutants and transformants (*ent*-kaurene overproducer), the compound was incorporated into the other *A. thaliana* mutants, which were unable to produce *ent*-kaurene. The *ent*-kaurene-exposed mutants recover the ability to produce gibberellins, thereby overcoming their gibberellin-deficient phenotype (Otsuka et al. 2004). These findings suggest that plants are able to take in volatile phytohormone precursors and convert them to their active forms.

13.3.2 Glycosylation of Volatile Alcohols

Conversion of (*Z*)-3-hexenol to its corresponding glycoside has been reported, which has implications for plant–plant communication (Sugimoto et al. 2014). In a non-targeted metabolomic analysis of volatile-exposed tomato, we found that the plants accumulated (*Z*)-3-hexenyl vicianoside when they were exposed to volatiles emitted from herbivore-infested conspecifics (Sugimoto et al. 2014). Interestingly, the aglycone of (*Z*)-3-hexenyl vicianoside, (*Z*)-3-hexenol, is one of the green leaf volatiles commonly found in herbivore-infested plants. When plants were exposed to labelled (*Z*)-3-hexenol, the accumulated (*Z*)-3-hexenyl vicianoside was labelled. Therefore, the origin of the aglycone was confirmed as the exogenous volatile compounds. Additionally, we observed that (*Z*)-3-hexenyl vicianoside functioned as a defence compound against herbivores. These results indicate that the plants could incorporate the airborne (*Z*)-3-hexenol and convert it to (*Z*)-3-hexenyl vicianoside as a form of protection from future attack by herbivores.

To assess whether the glycosylation of volatiles is specific to tomato plants, the presence of (*Z*)-3- hexenyl glycosides was examined in 24 plant species from 10 randomly chosen families (Table 13.1). Because the accumulation of (*Z*)-3-hexenyl glycoside was observed in all species, we concluded that glycosylation is likely commonly used among plants to perceive exogenous volatile compounds. Although a detailed study on the functions of different forms of glycosides is needed, glycosylation of volatiles has a role in plant communication (Sugimoto et al. 2014). Since the sugar moiety of (*Z*)-3-hexenyl glycosides is different among plant species, their functions might also differ. Moreover, there are differences in the aglycone moiety derived from volatiles. When *A. thaliana* plants were exposed to various volatile alcohols including aliphatic, terpene, and aromatic alcohols, they accumulated them in a glycosylated form (Table 13.2, Sugimoto et al. 2015). To verify the glycosylation process, it is necessary to identify the enzymes involved in glycosylation and examine their substrate preferences among the plant volatiles.

Glycosylation may be a common strategy to convert and store volatile compounds in plants. The glycosylated form of volatile alcohols has been observed in specific tissues (e.g. fruits) of plants that have not been exposed to volatiles (Tikunov et al. 2010), where they are believed to function as a source of flavours.

Table 13.1 List of plant families known to synthesize (*Z*)-3-hexenyl glycosides

Family	Species	Z3HexGlc	Z3HexVic	Z3HexPrim
Poaceae	*Oryza sativa*	+	+	+
	Sorghum sp.	+		+
	Triticum aestivum	+		
Cucurbitaceae	*Citrullus lanatus*	+	+	
	Cucumis melo	+	+	
	Cucumis sativus	+	+	+
	Momordica charantia	+	+	+
Fabaceae	*Lotus japonicus*	+		
	Phaseolus lunatus	+	+	
	Phaseolus vulgaris	+	+	+
	Trifolium repens	+	+	
Malvaceae	*Abelmoschus esculentus*	+	+	
Brassicaceae	*Arabidopsis thaliana*	+		
	Brassica rapa	+		
	Eruca vesicaria	+		
	Raphanus sativus	+		
Plantaginaceae	*Antirrhinum majus*	+		
	Plantago asiatica			+
Lamiaceae	*Melissa officinalis*	+		
Solanaceae	*Solanum lycopersicum*	+	+	+
	Solanum melongena	+	+	
	Nicotiana tabacum	+	+	
Asteraceae	*Arctium lappa*	+		+
Apiaceae	*Petroselinum crispum*	+		+

When the 24 species of plants from 10 families were exposed to green leaf volatiles, these plants converted them into the three different types of (*Z*)-3-hexenyl glycosides: (*Z*)-3-hexenyl glucoside (Z3HexGlc), (*Z*)-3-hexenyl vicianoside (Z3HexVic) and (*Z*)-3-hexenyl primeveroside (Z3HexPrim), depending on their ability to use the different sugar donors. +: detected; empty cell: under the detection level

In terms of the biosynthesis of flavour glycosides, genes for some glycosyltransferases have been isolated and identified. For example, genes for biosynthesis of phenylpropene triglycoside in tomato fruits (Louveau et al. 2011; Tikunov et al. 2013), terpene glycoside in kiwi fruits (Yauk et al. 2014) and terpene and (*Z*)-3-hexenyl glycosides in tea leaves (Ohgami et al. 2015) have been isolated. Homologues of these genes might also function in the processing of exogenous volatiles during plant communication.

13.3.3 Reduction, Esterification and Glutathionylation of Green Leaf Volatiles

Green leaf volatiles consist of six carbons and are derived from the oxygenation of fatty acids. They are emitted from almost all green plant tissues. These volatiles are

Table 13.2 List of volatile alcohols that could be converted into their glucosides in *Arabidopsis thaliana*

Volatile categories	Volatile compounds	Glucoside accumulation
Aliphatic	(*Z*)-2-pentenol	+
	(*Z*)-3-hexenol	+
	(*Z*)-3-heptenol	+
	(*Z*)-3-octenol	+
	(*Z*)-3-nonenol	+
Cyclic hydrocarbon	Cyclohexanol	+
Aromatic	Benzyl alcohol	+
Strait chain monoterpene	Linalool	+
	Geraniol	+
Cyclic monoterpene	Verbenol	+
	Perillyl alcohol	+
	Myrtenol	+

The Arabidopsis plants, which were exposed to 12 volatile alcohols from five structural categories, could convert them into their glucosides and accumulate them

synthesized at physically damaged regions and are commonly emitted from herbivore-infested plants (Matsui 2006; Arimura et al. 2009). The first volatile compound formed during green leaf volatile biosynthesis is (*Z*)-3-hexenal. A portion of (*Z*)-3-hexenal is reduced to yield (*Z*)-3-hexenol. Subsequently, a portion of (*Z*)-3-hexenol is further converted to (*Z*)-3-hexenyl acetate by a BAHD acyltransferase (D'Auria et al. 2007). This process also occurs when uninjured plants are exposed to (*Z*)-3-hexenal or (*Z*)-3-hexenol. After uninjured *A. thaliana* plants were exposed to labelled (*Z*)-3-hexenal, they started emitting labelled (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate (Matsui et al. 2012). The (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate were not derived from newly-synthesized (*Z*)-3-hexenal, but rather from the original volatile compounds. This was confirmed by the fact that only labelled (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate were detected (Matsui et al. 2012). These results indicate that airborne volatiles such as (*Z*)-3-hexenal are incorporated into plants and converted to other volatiles, such as (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate, through biosynthesis pathways.

The potential physiological roles of conversion have been investigated. (*Z*)-3-Hexenal might be produced to deter pathogenic pests in damaged plant regions because the compound is chemically reactive (Shiojiri et al. 2006). Its reactivity may also make it toxic to the plant itself (Matsui et al. 2012). Therefore, it is believed that excess reactive volatiles need to be reduced and esterified to detoxify the compounds.

In addition to reduction and esterification, glycosylation and glutathionylation are also involved in detoxification pathways (Mano 2012; Kim and Bowles 2004). Under conditions of oxidative stress, tobacco leaves accumulate the glutathione conjugate of hexenal (Davoine et al. 2006). Additionally, hexenyl glutathione was observed to accumulate in plant tissues (Kobayashi et al. 2011; Fedrizzi et al. 2012), and its abundance increased under stress conditions (Kobayashi et al. 2011). These findings suggest that glutathionylation is an alternative pathway to convert volatile compounds. Methacrolein, which is a reactive volatile, was converted to its glutathione conjugate in tomato plants exposed to methacrolein vapour (Muramoto et al. 2015). These studies show that various kinds of processing of green leaf volatiles may occur in exposed plants (Fig. 13.2).

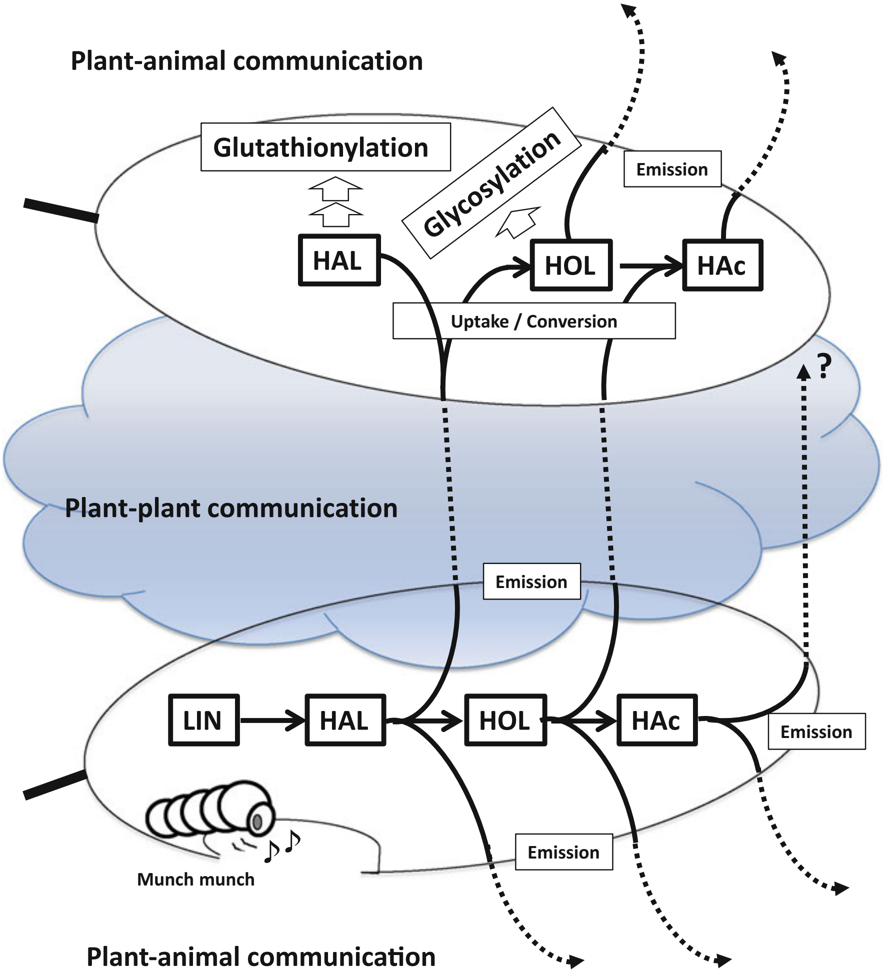

Fig. 13.2 Processing of green leaf volatiles in leaf tissue. Herbivore-infested leaves produce green leaf volatiles [mainly (*Z*)-3-hexenal (HAL), (*Z*)-3-hexenol (HOL) and (*Z*)-3-hexenyl acetate (HAC)] from polyunsaturated fatty acids through the phytooxylipin pathway. A portion of the volatiles is absorbed and taken in by neighbouring leaves. The volatiles in the neighbouring leaf are processed in the phytooxylipin pathway. At the same time, HOL molecules are converted into their glycoside form, and (*E*)-2-hexenal molecules, the converted form of HAL, are further converted into glutathione conjugate. They are accumulated within leaf tissues

13.4 Conclusions, Unanswered Questions and Perspectives

It is now widely accepted that plants have the ability to respond to airborne volatiles (for review: Yoneya and Takabayashi 2014). We herein summarized how plants take up volatile compounds and process them in their tissues as one of the pathways of volatile perception (see also Chap. 12). An intriguing question regarding the mechanisms involved in plant–plant communication is how plants respond differently to diverse volatile compounds (Kishimoto et al. 2005) and show particular responses to specific concentrations of volatile mixtures (Kikuta et al. 2011). One of the enzymes for the synthesis of (*Z*)-3-hexenyl vicianoside has been cloned and characterized using a molecular genetics approach (Sugimoto et al. unpublished). Determining the biochemical properties (e.g. substrate preference) of the identified enzyme and its homologs, paralogs and orthologs would in part explain how plants distinguish the various volatiles. To clarify the above questions, further studies on how plants incorporate and convert these compounds at the cellular level are needed. For example, in phytohormone responses, jasmonates were imported into cells through a transporter (Saito et al. 2015) and combined with a receptor complex to transmit signals (Thines et al. 2007). In the case of volatile compounds, however, it is unclear whether transporters or transporter-like mechanisms are involved. For processing volatiles, plants exposed to volatiles exhibit several cellular responses such as the depolarization of membrane potential, increased entry of calcium ions in the cytosol (Zebelo et al. 2012) and the induction or priming of defence gene expression (Arimura et al. 2000; Ali et al. 2013). These responses might be under the control of signalling pathways within cells. However, whether the specific processing of volatiles as mentioned above is mandatory for the signalling pathways has not been determined.

Characterizing the molecular mechanisms of volatile perception by plants in plant–plant communication would provide important clues that enable us to increase crop resistance to biotic stress through selective plant breeding. Furthermore, clarifying the ecological/plant physiological conditions under which plant species-specific plant–plant communication takes place (e.g. Kikuta et al. 2011) would also be important information for breeding. Comprehensive studies on plant–plant communication by plant physiologists and chemical and community ecologists would shed light on novel environmentally benign pest management strategies.

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Part IV
Synthesis and Future Directions

Chapter 14
Deciphering Chemical Language of Plant Communication: Synthesis and Future Research Directions

Robert Glinwood and James D. Blande

14.1 Synthesis

To decipher the chemical language of plant communication, we firstly need to investigate its effects on the recipients and to try and understand the selective forces that lie behind its evolution. Secondly, we need to translate the language itself, assigning meaning to the chemical compounds and blends that make up its words and sentences. Finally, we must know how the language is produced and received and the mechanisms of plant chemical production and perception. The knowledge and ideas presented in the chapters of this book show that we are making progress on all these points.

Imagine a community consisting of plants, herbivores, predators and parasitoids, pollinators, and a complex network of microorganisms. Chemical-mediated communication may play a role in almost every possible interaction between the members of this community. As well as advances in the understanding of plant–insect communication, communication between plants is now well established. For example, plant–plant volatile communication upon herbivore attack has been observed in more than 30 plant species, and 40 out of 48 studies of plant–plant communication via herbivore-induced plant volatiles (HIPVs) have found evidence of communication that affect herbivory (Li, Chap. 7; Karban et al. 2014a). Evidence is emerging that plant–plant volatiles can carry information not only on

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attack by antagonists but also about abiotic stress, e.g. salt (Lee and Seo 2014) and UV-C irradiation (Yao et al. 2011), and on potential competition and kin recognition (e.g. Karban et al. 2014b). Conversely, our understanding of the role of plant volatiles for interactions involving microorganisms (and microorganism volatiles for plants) is at a much earlier stage.

In this closing chapter, we attempt to outline common themes emerging from the contributed chapters and suggest some key topics where future research should focus.

14.1.1 Plant Communication in a Community Context

Great progress has been made in deciphering bipartite communication between plants and other organisms, but this book illustrates clearly the need to take a wider view encompassing the entire community of organisms. Much research on plant volatiles has concentrated on tritrophic interactions, often with plant protection in mind, but plant volatile communication influences community processes and, therefore, community structure. This becomes clear when one considers the myriad interactions that can potentially be mediated by plant volatiles that includes, but is not limited to, pollination, parasitism, predation, intra-guild predation, competition, interference, facilitation, associational resistance, priming and induction of defence and stress tolerance, allelopathy, learning/conditioning and niche partitioning.

In this regard, the impact of the fourth trophic level should not be overlooked, particularly its influence on biocontrol of pests (Poelman and Kos, Chap. 9). This is a relatively new research area that opens up the idea that hyperparasitoids can utilise plant cues and, by doing so, have a top-down impact on beneficial insects. HIPVs can also affect the behaviour of vertebrates, for example appearing to act as kairomones for birds (Mäntylä et al. 2008) and lizards (Stork et al. 2011). The study of HIPVs has been almost exclusively the realm of entomologists, so collaboration with zoologists specialising in other animal groups is needed to fully reveal their ecological roles. Communication between plants and the volatile-receiving community involve either individual chemical compounds or blends of volatiles. These chemicals can have numerous effects on community members, with potentially opposing effects on the emitting plant depending on the organism receiving the signal. In evolutionary terms, the key recipient in some cases could be the emitting plant itself (Guerrieri, Chap. 5).

Changes in plant volatiles are starting to be considered in the context of trait-mediated indirect effects (Pareja and Pinto-Zevallos, Chap. 3; Stam et al. 2014), where herbivore-induced volatiles interfere with another volatile-mediated interaction. Pareja and Pinto-Zevallos (Chap. 3) write that ‘An important avenue of research will be integrating the accumulated behavioural and chemical information with the conceptual framework of indirect effects in ecosystems’, and Meiners (Chap. 6) notes ‘Including chemical diversity in biodiversity research is an emerging issue in ecosystem function research’. Floral scents are diverse and multifunctional, being involved not only in pollinator attraction but also repellence of antagonistic species and regulation of interactions with microorganisms (Junker, Chap. 11).

One should also note that organisms that can both affect and be affected by plant volatiles are not limited to plants, arthropods and vertebrates, but include a whole microbiological world that is so far hardly explored. Knowledge on the importance of plant volatiles below ground lags behind knowledge from the above ground environment. However, in the absence of sunlight, chemical information could have an even greater value belowground. Microorganisms can have a major impact on plant volatile blends (van Dam et al., Chap. 8), for example phyllosphere microbiota can significantly influence plant terpene emissions (Peñuelas et al. 2014). Volatiles released by microorganisms associated with plants can reprogram plant physiology and behaviour, leading to both positive and negative outcomes for the plant (van Dam et al., Chap. 8).

Recent advances have shown, for example, that without physical contact, bacteria can dramatically alter the development of the plant's root system with effects ranging from death to a sixfold increase in biomass. New roles for known substances are likely to emerge, for example indole, highlighted as a within—and between—plant signal (Erb et al. 2015), is also produced by plant growth promoting rhizobacteria (PGPR) and can promote plant growth. These advances open up the intriguing concept of context in plant communication; if plants respond in different ways to the same chemical cue what is underlying those different responses? In human communication, context is said to be everything; it shapes the meaning of content. While obviously not directly comparable to human communication, observations that plants might not be merely mechanically responding to one chemical in the same way in all instances does lend credence to the possibility that there is a contextual dimension in the plant communication process. Another emerging field is floral microbial ecology, and it is important to understand that the evolution of floral scents has probably been affected by selection not only from mutualists but also from microorganisms (Junker, Chap. 11; Junker and Tholl 2013).

Much of what we know about plant volatile-mediated interactions comes from studies in temperate, agricultural or forest systems. For a fundamental understanding of the importance of plant volatile communication, not least at the community level, more studies on tropical systems are needed. As Pareja and Pinto-Zevallos (Chap. 3) note '(tropical) regions are at the forefront of global efforts to stem the loss of biodiversity and ecosystem services, so focus on these systems is long overdue'. Within such ecosystems, it could be postulated that the information provided by chemical signals and cues is more important than for many more homogeneous ecosystems, although the alternative argument could be that the chaotic interplay of chemically diverse plants could render chemical signals largely ineffectual. At this point we simply do not know.

14.1.2 Signal Versus Noise

Considering the importance of plant chemical-mediated interactions for community processes inevitably leads to the question of specificity of the chemical signal and the ability of receivers to discern the information in a complex and dynamic environment. The biologically relevant cues may consist of single substances, but we now know that, in many cases, specific blends of compounds are important. These cues must be interpreted against a chemical background that is likely to become more complex with increasing organismal diversity. Meiners (Chap. 6) notes that laboratory studies that simulate the field conditions are needed, since most studies on arthropod responses to plant volatile diversity have been done in small scale olfactometer experiments, which are inadequate in this context.

In addition to the chemical background, the nature of the cue itself may be dynamic, changing according to both biotic and abiotic factors. For example, abiotic stresses such as temperature and drought can modify both constitutive and induced volatiles. These effects can then impact on biotic interactions. For example, there is evidence that drought can prime volatile defences upon biotic attacks (Copolovici et al. 2014). Diurnal and seasonal rhythms of volatile release are linked not only to abiotic factors but can be synchronised to biotic interactions in ways that are adaptive for the plant (Schuman, Chap. 1). Recently, evidence that the circadian clock directly regulates floral volatiles has been reported (Yon et al. 2016; Fenske et al. 2015).

Plant communication depends on the fidelity of a signal from an emitting organism to a receiver, which can be complicated immensely when plants are attacked by more than one organism or affected by multiple stress factors (Pareja and Pinto-Zevallos, Chap. 3). There is a great complexity and asymmetry in plant responses to multiple stresses. VOC emission under dual infestation may not merely be a combination of the VOCs induced by individual stressors, but can have its own unique profile that may carry specific information to a receiver (Pierre et al. 2011).

'Private channels' do seem to exist (Borges, Chap. 10; Junker, Chap. 11), in which interactions are restricted to particular pairs of species, but even here this communication occurs against a complex chemical background generated by the entire community. The volatile chemicals emitted by flowers and structuring the interactions with pollinators are often emitted by plants in other contexts, which brings into focus the issue of specificity and how it is encoded. The chapter by Borges (Chap. 10) provides a complete update to the brood-site pollination mutualism of figs and fig wasps, which is arguably the best studied mutualism of this type.

This book illustrates the dynamic nature and sensitivity of plant volatile blends, and their responsiveness to abiotic as well as biotic factors. It is therefore not surprising that plant volatile communication may be vulnerable to factors associated with climate change. Copolovici and Niinemets (Chap. 2) report how release of terpenoids and green leaf volatiles, compounds shown to affect multiple trophic interactions, can be affected by factors associated with climate change. It should also be noted that plant volatile emissions can contribute to atmospheric pollution and global climate regulation (Arneth and Niinemets 2010). Reactions between atmospheric pollutants and plant volatiles create new products, which may further increase noise and reduce the efficiency of plant communication (Li, Chap. 7).

Understanding these processes will be critical both to our fundamental understanding of plant chemical communication, but also our possibilities to make use of it in practice, in integrated plant protection for example. New types of collaboration will be needed, for example between biologists and atmospheric chemists and physicists.

It is worth noting that, to successfully study the question of signal versus noise in plant chemical communication, robust and sensitive methods will be needed. Fortunately, methods for volatile collection are advancing all the time, with enhanced sensitivity in the laboratory and an increasing ability to track plant volatile emissions in real time at the community and landscape scale (Misztal, Chap. 4). Within the fields of atmospheric chemistry and environmental science, technology has been developed to measure volatile chemicals over a large area with a high degree of spatial and temporal resolution (Misztal, Chap. 4). Coupling this technology to the most advanced methods for tracking insects and vertebrates opens up new possibilities to overlay maps of volatile plumes and animal orientation. It is clear that coupling these technologies will require a great deal of development, but the basic components are in place to make major inroads into distinguishing signal from noise. One of the most challenging tasks for chemical ecologists has been to extrapolate mechanisms elucidated in the laboratory at a small scale to the situation in the field. Meiners (Chap. 6) and Guerrieri (Chap. 5) both call for efforts to increase experimental scale; collaboration between atmospheric chemists and chemical ecologists could lead to major advances in our understanding of volatile-mediated communication processes.

14.1.3 Search for the Plant 'Nose'

The field of chemical ecology has been heavily biased towards studies on plants as a signal provider, emitting volatiles that act as signals or cues for other members of the community, but recent reports that plants can listen-in, or eavesdrop, on insect pheromones (Helms et al. 2013, 2014) as well as neighbouring plants and microorganisms really fuel the need to understand how plants detect and process the information encoded in volatile chemicals. Two chapters in this book focus on how plants may perceive volatile cues (Sugimoto et al., Chap 13) and the early, rapid signalling events that they trigger (Zebelo and Maffei, Chap. 12). The quest for understanding of plant volatile perception has been somewhat of a holy grail for the research field for a number of years, and these authors present cutting edge knowledge on this vital question.

Plant electrophysiology is emerging as a means to examine early plant responses to damage and volatile cues (Zebelo and Maffei, Chap. 12). Change in electrical potential as an early event in plant response to stress may represent a very rapid mode of internal signalling. It is as yet unclear whether it functions as a primary or confirmative signal. A key breakthrough has been development of a system to perform electrophysiology in intact living plants. Using this system, it was shown that VOCs emitted by herbivore-damaged tomato plants trigger a plasma transmembrane potential depolarisation in mesophyll cells of neighbouring, undamaged tomato plants (Zebelo et al. 2012).

An important breakthrough in the search for the plant 'nose' is recent understanding of a potential detection/perception mechanism involving conversion of volatiles into defensive metabolites or active signalling molecules by glycosylation (Sugimoto et al., Chap. 13). Plants can incorporate airborne (*Z*)-3-hexenol and convert it to (*Z*)-3-hexenyl vicianoside as a form of protection from a future attack by herbivores (Sugimoto et al. 2014). Glycosylation is likely to be commonly used among plants to perceive exogenous volatile compounds. The significance of this process under natural conditions requires further research, but these recent studies promote this mechanism as a foundation for further research. Specific mixtures of volatiles appear critical to plant responses, which will be essential to understand in the future.

Understanding here is in its infancy, but we anticipate a number of key breakthroughs including mapping of the molecular and physiological mechanisms and an explanation of how plants might capture detailed information coded by specific blends of volatile compounds. In recent times, high throughput methods for DNA and RNA sequencing have become more accessible and affordable. The use of these and other developing molecular tools in carefully manipulated experiments involving collaboration between chemical ecologists, plant physiologists and bioinformaticians will open possibilities to understand the molecular mechanisms of plant volatile detection and response.

14.2 Future Research Directions

To continue the scientific progress reported in the chapters of this book and advance understanding of plant volatile communication to the next level, we will increasingly need to work in a transdisciplinary way, and to think broadly about the importance of this communication at the level of communities, rather than individuals. Below we highlight some of the key points that should guide future research in this field. The list is not exhaustive, and further valuable suggestions can be found in each chapter.

- Adopt a community approach, since volatiles can affect the entire web of ecological interactions and may influence community composition and function.
- Integrate functional, evolutionary, physiological and ontogenetic levels.

- Explore the ecological roles that plant VOCs play in more complicated ecological settings, including those in tropical regions and in non-agricultural systems.
- Tackle the challenge of exploring volatile interactions involving plants and microorganisms underground, under natural as well as laboratory conditions.
- Understand how pollution can both influence plant volatile production and impact the efficiency of communication by released compounds.
- Describe novel mechanisms of plant perception of volatile cues and whether they are transduced by receptor-mediated processes or simply interact with plant membranes and initiate signal transduction pathways.
- Investigate how plants convert compounds at the cellular level and characterise the molecular mechanisms of volatile perception.

Although the book focuses mostly on ecological interactions, there is great potential to exploit plant volatiles in management of arthropod pests using, for example, attractants, repellents and defence elicitors (Guerrieri, Chap. 5). In this case, there is a need for large scale field studies, which are often avoided due to the time and resources needed. However, practical application of plant chemical communication depends on a thorough fundamental understanding of the interactions. This will only be fully attained when we can deal with the daunting level of complexity found in natural systems. Borges (Chap. 10) concludes ‘The need of the hour is collaboration between various disciplines—ecology, evolution, chemistry, atmospheric science, fluid dynamics, behaviour and neurobiology’. It is time for a broader, community level approach to the study of plant volatile communication, in which these types of collaboration help us decipher the chemical language of plant communication.

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