Stop and smell the fungi: Fungal volatile metabolites are overlooked signals involved in fungal interaction with plants

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Abstract

Diverse fungi are intimately associated with plants, and molecules secreted from both the plant and fungal sides play critical roles in the establishment of their associations and affect plant growth and health. Through evolutionary arms races or strategic alliances with plants, fungi and other microbes have invented a bewildering array of secreted molecules to parasitize or communicate with plants. Research empowered by omics data and tools has greatly advanced understanding of the nature, role and mechanism of action of many secreted fungal proteins that affect plants directly or indirectly. However, available information about fungal volatile metabolites with similar functions is quite limited. Through this review, we aim to stimulate expeditions to this vastly under-explored frontier of fungal chemical ecology.

Keywords

Chemical ecology; Inter-organismal signals; Plant-fungal interaction; Plant fitness; Semio-compounds; Volatilome

1. Introduction

In a rather simplistic and anthropocentric manner, plant-associated microbes can be classified as ‘beneficial, detrimental, or unknown’ based on their effects on plant growth and health. Microbes secrete diverse classes of molecules that directly or indirectly affect plant growth, development, productivity and overall health (Bednarek et al., 2010). Characterization of the nature, synthesis, function and mechanism of action of such molecules is critical for understanding how plants and microbes interact and how their interactions have evolved. One well documented class of secreted molecules includes phytohormones, such as indole-3-acetic

1 acid, cytokinins and gibberellins (Chanclud et al., 2016; Gutierrez-Manero et al., 2001; Loper
2 and Schroth, 1986; Macdonald et al., 1986; Timmusk et al., 1999), and molecules that mimic
3 phytohormones, such as coronatine produced by *Pseudomonas syringae* (Zheng et al., 2012) and
4 methylated cytokinins released by *Rhodococcus fascians* (Radhika et al., 2015). Diverse and
5 often inter-connected roles of the hormones in regulating many physiological and developmental
6 processes, including defense responses, likely presented evolutionary opportunities for plant-
7 associated microbes to hijack or manipulate such processes in plants by releasing one or more
8 hormones or their structural or functional analogs. Plant pathogens secrete assorted virulence
9 factors (e.g., proteins, small RNAs, and metabolites) into the plant-pathogen interface and plant
10 cells to disarm or manipulate plant defense machineries so as to facilitate pathogenesis
11 (Chanclud et al., 2016; Collemare and Lebrun, 2011; Deslandes and Rivas, 2012; Rovenich et al.,
12 2014; Weiberg et al., 2014; Zheng et al., 2012). The manipulation of plant defense via the
13 secretion of such molecules is not limited to pathogens. Establishment of the symbiotic
14 associations with rhizobia and mycorrhizal fungi also requires suppression of host defense
15 responses, because like pathogens, these beneficial microbes also display pathogen-associated
16 molecular patterns (PAMPs), which activate basal defense responses termed PAMP triggered
17 immunity (PTI) (Evangelisti et al., 2014; Jones et al., 2007; Plett and Martin, 2011). These
18 symbiotic associations also involve specific secreted signals, from both the plant and microbe
19 sides, in order to initiate and coordinate appropriate developmental and growth changes required
20 for the establishment of their partnership (Harrison, 2005; Jones et al.,
21 2007; Perret et al., 2000; Venturi and Keel, 2016). Plant growth-promoting rhizobacteria (PGPR)
22 and fungi (PGPF) enhance plant growth and defense by influencing the plant physiology via the

1 use of diverse secreted molecules (Bakker et al., 2013; Kundan et al., 2015; Pieterse et al., 2014;
2 Shores et al., 2010; Venturi and Keel, 2016).

3
4 Accordingly, there have been concerted efforts to identify and functionally characterize
5 microbial secretomes (Bednarek et al., 2010; Chisholm et al., 2006; Hogenhout et al., 2009). In
6 addition to the fundamental significance, increasing need to sustain crop growth and health
7 without relying so heavily on pesticides and fertilizers has also driven research in this field
8 (Bednarek et al., 2010). Enhanced knowledge on the microbial secretomes and their mechanisms
9 of action should help facilitate the development of bio-based strategies for enhancing crop
10 production and protection. However, in comparison to the copious available data on secreted
11 microbial proteins, relatively little is known about the nature and role of secreted microbial
12 metabolites, especially those that are volatile, in plant-microbe interactions. Both fungi and
13 bacteria produce diverse volatile (or semi-volatile) organic compounds (VOCs), which comprise
14 multiple classes of low-molecular weight lipophilic metabolites and their byproducts that
15 vaporize at normal temperatures and pressures (Lemfack et al., 2014). VOCs produced by fungi
16 appear to have similar functions as those emitted from animals and plants, and play a critical role
17 in ecological fitness, such as inhibition of competing organisms, coordination of growth and
18 development within species, and communication with other neighboring organisms (Bailly and
19 Weisskopf, 2012; Bitas et al., 2013; Morath et al., 2012; Rohlfs and Churchill, 2011). However,
20 key questions such as which fungal VOCs confer such functions, how they manipulate other
21 organisms, and how their synthesis occurs and is regulated, remain mostly unanswered.
22 Systematic exploration of the diversity and function of fungal VOCs and their mechanisms of
23 action will advance understanding of fungal biology and ecology in association with plants,

1 animals and other microbes. This review focuses on the role of fungal VOCs in affecting plant
2 growth, development, stress resistance, or combinations of these traits. Our goal is to emphasize
3 critical knowledge gaps with hopes to stimulate concerted efforts to address them. We also
4 discuss several technical challenges, which must be overcome in order to expedite explorations
5 of VOC-mediated plant-fungal interactions.

6 7 **2. Volatile signals play vital roles within and across multiple kingdoms**

8 Considering that water is not consistently readily available in nature, it is unlikely that microbes
9 would rely on water as the sole medium for secreted molecule-mediated interactions with
10 neighboring organisms. Because volatile metabolites can travel through water, air and porous
11 soils, they are more versatile than water-soluble signals and can function as both short- and long-
12 range signals. Animals, plants and microbes have evolved the ability to produce and recognize
13 volatile metabolite signals as a means of communication, and volatile-mediated signaling
14 processes serve vital ecological functions (Bitas et al., 2013; Herrmann, 2010; Li and Liberles,
15 2015; Schenkel et al., 2015). In the absence of plant VOCs that function as homing cues to
16 pollinators and seed dispersers (Baldwin, 2010; Herrmann, 2010), the terrestrial landscape and
17 its inhabitants would look quite different. Some plants even mimic fungi by releasing a mixture
18 of VOCs that are commonly emitted by mushrooms to attract certain insects for pollination
19 (Kaiser, 2006). Without volatile cues released from plants parasitized by pests, neighboring
20 plants would not receive an advanced warning of imminent threats, and parasitoids of insect
21 herbivores would struggle to find their preys (Baldwin, 2010). If animals could not associate
22 specific volatile compounds with food, mates or threats, they would not likely have survived
23 very long or prospered. Similarly, microbial communities would likely become dysfunctional in

the absence of volatile communications, as microbial VOCs are thought to be involved in antagonism, mutualism, and intra- and inter-species regulation of cellular and developmental processes (Bitas et al., 2013; Schmidt et al., 2015). However, relatively little information is available regarding bacterial VOCs as signals, and even less is known about fungal VOCs.

Diversity of fungal VOCs and their potential functions

Although some fungi, such as truffles (*Tuber* spp.), have received a lot of attention and have a long history of volatile research (Splivallo et al., 2011), studies on the nature and role of VOCs have been limited to a very small subset of fungi (Bitas et al., 2013; Hung et al., 2015; Morath et al., 2012). The online database that archives identified bacterial and fungal VOCs, mVOC (Lemfack et al., 2014), lists only approximately 300 known fungal VOCs, including (in descending order by abundance) alcohols, benzenoids, aldehydes, alkenes, acids, esters, terpenoids, and ketones. Whereas more than 1,700 volatile compounds have been identified from plant flowers alone (Baldwin, 2010). Given the immense untapped diversity of fungi (Blackwell, 2011), we have likely not even scratched the surface of fungal VOC diversity.

Despite the limited sample size, investigations have focused on the following functions of fungal VOCs: a) chemical weapon against other microbes, b) signal for intra- and inter-species communications, and c) regulator of plant growth and/or stress resistances. Some VOCs produced by species such as *Muscodor* spp., *Trichoderma* spp., *Pleurotus ostreatus* and *Epichloë* sp. exhibited anti-bacterial and/or anti-fungal activity (Beltran-Garcia et al., 1997; Mitchell et al., 2010; Steinebrunner et al., 2008a; Zhang et al., 2014). Fungal VOCs also appear to function as intra- and inter-organismal signals. One such VOC, 1-octen-3-ol (Fig. 1), is commonly produced

by many fungi and appears to control spore production and germination (Chitarra et al., 2004; Nemcovic et al., 2008). Some rust fungi attract insects for spore dispersal by forming shapes (*Puccinia arrhenatheri*) and VOCs (*P. punctiformis*) that enable them to mimic flowers (Kaiser, 2006). Fungal VOCs that have been shown to attract insects include 1-octen-3-ol, methyl (Z)-3-methyldodec-2-enoate and chokol K (Fäldt et al., 1999; Schiestl et al., 2006; Splivallo et al., 2011; Steinebrunner et al., 2008b). Additional information regarding antimicrobial activity and insect attraction by fungal VOCs can be found in the recent reviews (Bitas et al., 2013; Hung et al., 2015; Kanchiswamy et al., 2015a). A small number of known fungal VOCs that influence plant growth and/or stress resistances (Table 1 and Fig. 1) are the focus of this review. Nevertheless, the first two functions attributed to fungal VOCs also likely influence plant health and fitness indirectly by manipulating the structure and emergent properties of microbial communities associated with plants.

Bacterial VOCs as regulators of plant growth and stress resistance

Prior to overviewing the current status of research on how fungal VOCs affect plants, we must first review a few key findings from bacterial VOC research. This is because initial attempts to identify and characterize fungal VOCs as potential regulators of plant growth and defense were stimulated by earlier studies of bacterial VOCs with such functions. Much more information is available about how bacterial VOCs affect plants, and some aspects of the molecular mechanism underlying the VOC-mediated interaction between plants and bacteria are similar to those between plants and fungi (Audrain et al., 2015; Bailly and Weiskopf, 2012; Bitas et al., 2013; Farag et al., 2013; Kanchiswamy et al., 2015a). The physiological response of plants to bacterial VOCs and the underlying molecular mechanisms involved have been primarily elucidated using

1 *Arabidopsis thaliana*, due to diverse resources available for this model plant. Ryu et al. (2003)
2 first demonstrated the enhancement of *A. thaliana* growth by the VOCs produced by two strains
3 of plant growth promoting rhizobacteria (PGPR), *Bacillus subtilis* GB03 and *B.*
4 *amyloliquefaciens* IN937a. In order to elucidate their underlying mechanism of action, two
5 complementary approaches have been utilized: a) analysis of *A. thaliana* gene expression
6 patterns upon exposure to PGPR VOCs or synthesized candidate volatile compounds, and b)
7 analysis of the growth response of mutant *A. thaliana* defective in various hormonal signaling
8 pathways to VOCs. Up-regulation of several key enzymes in auxin and ethylene biosynthesis and
9 down-regulation of abscisic acid (ABA) signaling were ascribed to *B. subtilis* GB03 VOC-
10 mediated growth enhancement of *A. thaliana* (Kwon et al., 2010; Zhang et al., 2008b, 2007).
11 Growth response of *A. thaliana* hormonal signaling mutants to rhizobacterial VOCs suggested the
12 involvement of ethylene and cytokinin signaling pathways (Huang et al., 2012; Ortíz-Castro et
13 al., 2008; Ryu et al., 2003). Bacterial VOCs that exhibited plant growth-promoting activity
14 include 2,3-butanediol, acetoin, dimethyl hexadecylamine, indole, and 2-pentylfuran (Bailly et
15 al., 2014; Ryu et al., 2003; Velázquez-Becerra et al., 2011; Zou et al., 2010). Using a
16 combination of genetic and biochemical approaches, Bailly et al. (2014) demonstrated that indole
17 works through the auxin signaling pathway to enhance plant growth. In contrast, other bacterial
18 VOCs, such as 2-phenylethanol and tridecane, inhibited plant growth (Kai et al., 2010; Lee et al.,
19 2012). Exposure of *A. thaliana* to inhibitory VOCs emitted by *Serratia plymuthica* and
20 *Stenotrophomonas maltophilia* caused induced expression of several genes enriched for W-box
21 motifs in their promoter and stress-inducible transcriptional factor genes (Wenke et al., 2012).
22 Intriguingly, dimethyl disulfide inhibited *A. thaliana* growth (Kai et al., 2010), but under sulfur-

depleted conditions, it enhanced *Nicotiana attenuata* growth presumably by serving as a sulfur source (Meldau et al., 2013).

Some bacterial VOCs also helped protect plants from (a)biotic stresses. For instance, 2,3-butanediol induced drought tolerance in *A. thaliana* through an salicylic acid (SA)-dependent signaling pathway (Cho et al., 2008) and also elicited induced systemic resistance, making exposed plants more resistant to *Erwinia carotovora* subsp. *carotovorum*, a necrotrophic bacterial pathogen (Ryu et al., 2004). Upon exposure to VOCs produced by *B. subtilis* and *Pseudomonas simiae*, *A. thaliana* and soybean seedlings grew better under salt stress, and the underlying mechanism seems to include the up-regulation of several enzymes involved in osmotic stress response (Vaishnav et al., 2015; Zhang et al., 2010, 2008a). Furthermore, tridecane produced by *Pseudomonas polymyxa* elicited a strong plant defense against *P. syringae* pv. tomato via the SA- and jasmonic acid (JA)-signaling pathways (Lee et al., 2012).

3. Effects of fungal VOCs on plants

VOCs that enhance plant growth

Plants have evolved to form alliances with many fungi to ensure healthy growth (Evangelisti et al., 2014). Plant growth promoting fungi (PGPF), which are soil saprophytes, symbionts or plant endophytes, have been reported to enhance seed germination, as well as plant growth and (a)biotic stress tolerance (Shivanna et al., 1996; Waqas et al., 2012). One or more of these effects have been suggested to occur via several mechanisms, such as fungal production of phytohormones, increased mobilization of mineral nutrients and water, causing induced resistance, and direct suppression of pathogen growth (Altomare et al., 1999; Brimner and

Boland, 2003; Contreras-Cornejo et al., 2014, 2009; Jeffries et al., 2003; Shivanna et al., 1996).
 However, the potential involvement of fungal VOCs in benefiting associated plants has been
 largely unexplored. A bicyclic sesquiterpene, β -caryophyllene (Fig.1), produced by the PGPF
 strains *Talaromyces wortmannii* FS2 and *Fusarium oxysporum* MSA 35, enhanced the growth of
 lettuce (Minerdi et al., 2011) and Komatsuna plants (Yamagiwa et al., 2011). However, *A.*
thaliana and poplar were not affected by β -caryophyllene, suggesting that its growth promoting
 effect depends on hosts (Ditengou et al., 2015). Exposure to a mixture of 2-methyl-propanol, 3-
 methyl-butanol, methacrylic acid and isobutyl acetate (Fig.1), VOCs produced by *Phoma* sp.
 GS8-3, caused a slight but significant increase in the fresh weight of tobacco (Naznin et al.,
 2013). VOCs from *Trichoderma viride*, a biocontrol agent, boosted *A. thaliana* shoot fresh
 weight and total chlorophyll content (Hung et al., 2013). Many strains of *F. oxysporum*, a
 cosmopolitan soil-borne species complex, produced VOCs that enhanced both shoot and root
 growth in *A. thaliana* and tobacco (Bitas et al., 2015). Responses of *A. thaliana* mutants and a
 GUS reporter line suggested that auxin signaling and transport is critical for causing these effects
 (Bitas et al., 2015).

Symbiotic association between ectomycorrhizal (ECM) fungi and the roots of most tree species
 is essential for forest health and productivity (Smith and Read, 2008). Exchange of various
 signals between them through multiple stages of association seems to modulate growth and
 development at both sides and suppress plant defense in order to permit root colonization by
 fungal hyphae (Plett and Martin, 2011). Some ECM VOCs might participate in this
 evolutionarily fine-tuned signaling process. A volatile sesquiterpene emitted by *Laccaria*
bicolor, (-)-thujopsene (Fig. 1), induced lateral root formation in both *A. thaliana* and poplar

plants (Ditengou et al., 2015). The lateral root induction by (-)-thujopsene in *A. thaliana* does not appear to involve auxin signaling. Truffles produce both auxin and ethylene. Application of the ethylene precursor L-methionine further stimulated root branching of their host *Cistus incanus*, suggesting that ethylene produced by truffles acts synergistically with auxin to regulate plant root development (Splivallo et al., 2009). VOC-mediated communication between ECM fungi and plants does not appear host-specific, since ECM VOCs caused similar effect on *A. thaliana* and other non-host plant species (Ditengou et al., 2015; Splivallo et al., 2009).

VOCs inhibitory to plant growth

Fungal VOCs can also negatively impact plant growth (Table 1), presumably by interrupting the plant plasma and organellar membranes or interfering with enzyme activity (Junker and Tholl, 2013). The VOCs 1-octen-3-ol, 3-octanol, and trans-2-octenal (Fig.1), emitted by truffle fruiting bodies, caused bleaching of *A. thaliana* leaves as a result of an oxidative burst, which is commonly associated with (a)biotic stress response (Splivallo et al., 2007). VOCs emitted by cereal fungal pathogens *Cochliobolus sativus* and *Fusarium culmorum* inhibited barley root growth (Fiers et al., 2013). Similarly, VOCs produced by *Muscodora yucatanensis*, an endophytic fungus isolated from a tree, inhibited growth of amaranth, tomato, and barnyard grass (Macías-Rubalcava et al., 2010). Evaluation of the effect of 23 commonly produced fungal VOCs on *A. thaliana* seed germination and vegetative growth showed that aldehydes and ketones displayed inhibitory activity (Hung et al., 2014). Some fungal VOCs modulated plant growth differently at different concentrations, suggesting the importance of dose for resulting effect. For example, 6-pentyl- α -pyrone (6PP) (Fig. 1) produced by *Trichoderma* spp. enhanced *A. thaliana* growth and increased root biomass presumably by affecting auxin transport and signaling as well as the

ethylene-response modulator EIN2 (Garnica-Vergara et al., 2015). However, when tested at a higher concentration, 6PP inhibited *A. thaliana* growth (Kottb et al., 2015). Similarly, 1-hexanol, which is produced by many bacteria and fungi, enhanced plant growth at low concentrations, but growth inhibition was observed at high concentrations (Blom et al., 2011; Splivallo et al., 2007).

VOCs as elicitors for increased resistance to (a)biotic stress

Both PGPR and PGPF have been extensively studied for their ability to cause induced systemic resistance (ISR), which primes the whole plant for enhanced defense against subsequent attacks by a variety of plant pathogens (Bakker et al., 2013; Pieterse et al., 2014; Shores et al., 2010).

The onset of ISR requires microbial elicitors (Pieterse et al., 2014). Great progress has been made in identifying such elicitors, which include microbe associated molecular patterns, siderophores, some antibiotics, *N*-acyl homoserine lactones, certain enzymes and peptides (De Vleeschauwer and Höfte, 2009; Djonovic et al., 2007). Certain fungal VOCs have been shown to cause ISR or similar forms of induced resistance. For example, VOCs produced by PGPF *Ampelomyces* spp. and *Cladosporium* spp. reduced disease symptoms in *A. thaliana* infected with *P. syringae* pv. tomato (Naznin et al., 2014). Three compounds, including *m*-cresol, methyl benzoate and 3-octanone (Fig.1), were identified as candidates responsible for the observed effect (Naznin et al., 2014). Evaluation of defense signaling mutants and expression patterns of *A. thaliana* defense-related genes suggested that ISR induced by *m*-cresol and methyl benzoate requires both the SA- and JA-dependent signaling pathways but works independent of the ethylene-dependent signaling pathway (Naznin et al., 2014). Additionally, some fungal VOCs that affect plant growth also appear to enhance resistance against pathogens. For instance, β -caryophyllene, which promoted lettuce growth, enhanced *A. thaliana* defense against

1 *Colletotrichum higginsianum*, a hemibiotrophic fungal pathogen (Minerdi et al., 2011;
2 Yamagiwa et al., 2011). Resistance of *A. thaliana* against *Botrytis cinerea*, a necrotrophic fungal
3 pathogen, was increased upon exposure to 1-octen-3-ol, a VOC that inhibited plant growth, and
4 the enhanced resistance requires the JA-dependent signaling pathway (Kishimoto et al., 2007;
5 Splivallo et al., 2007). Application of 6PP, which inhibited *A. thaliana* growth, also enhanced its
6 resistance against *Alterania brassicicola* and *B. cinerea* via the SA-dependent signaling pathway
7 (Kottb et al., 2015).

8 9 **Potential roles of the VOC-mediated manipulation of plants in fungal ecology**

10 The rhizosphere serves as the plaza and battle ground for soil inhabitants, and porous soils likely
11 mediate microbial VOC-mediated intra- and inter-organismal communications (Leff and Fierer,
12 2008; Piechulla and Degenhardt, 2014; Schenkel et al., 2015; Wenke et al., 2010). As outlined
13 above, VOCs produced by soil-borne fungi have been shown to enhance plant growth and/or
14 defense against (a)biotic stress. Since microbial fitness is likely linked to the fitness of
15 associated plants, one potential benefit of producing such VOCs can be found in their main
16 ecological niche, the rhizosphere. The rhizosphere offers rich nutrient sources for microflora, in
17 the forms of root exudates, dead root cells, and soil nutrients mobilized by metabolites and
18 enzymes secreted by plants and microbes. Increased nutrient availability due to enhanced root
19 growth would certainly help microbial growth. Enhancement of plant resistance to abiotic stress
20 would also likely help protect this fungal habitat under unfavorable environmental conditions.
21 Accordingly, the ability to boost plant growth and health by VOC production might functions as
22 a key trait for fungal rhizosphere competency – the ability to colonize and grow in association
23 with plant roots. Additional benefits may come from root developmental changes caused by

1 fungal VOCs. For instance, root penetration by *F. oxysporum* occurs primarily through the
2 meristematic region of primary and lateral roots (Czymmek et al., 2007; Turlier et al., 1994).
3 Increased formation of lateral roots induced by *F. oxysporum* VOCs (Bitas et al., 2015) could
4 facilitate infection by helping create more potential entry points to the vascular system.
5 Similarly, stimulated lateral root growth by VOCs from ECM fungi *L. bicolor* and truffles
6 suggests that their VOCs function as signals for the establishment of symbiosis (Ditengou et al.,
7 2015; Splivallo et al., 2009). Due to competition for nutrients and space in and around plants,
8 microbes also secrete a diverse array of proteins and metabolites as weapons against competing
9 microbes. Certain fungal VOCs may also participate in inter-species competition as anti-
10 bacterial and anti-fungal weapons. For example, VOCs produced by a biocontrol strain of *F.*
11 *oxysporum*, MSA 35, impaired mycelial growth of pathogenic *F. oxysporum* strains and
12 suppressed the expression of virulence genes in *F. oxysporum* f. sp. *lactucae* (Minerdi et al.,
13 2009). VOCs emitted by *M. yucatanensis* displayed fungistatic effect on many soil-borne fungi
14 (Macías-Rubalcava et al., 2010). A meta-analysis of known VOCs produced by plant roots and
15 those from bacteria and fungi commonly associated with roots revealed VOCs that are
16 potentially involved in belowground polytrophic interactions (Schenkel et al., 2015).

18 **4. Where to go from here and what lies ahead?**

19 The term “volatilome” (Maffei et al., 2007) sums up the need for and importance of
20 systematically exploring biogenic VOCs as intra- and inter-organismal signalings. As described
21 here and in previous reviews (Bitas et. al., 2013; Hung et al., 2015; Morath et al., 2012), some
22 fungal VOCs seem to participate in several critical processes within and across the kingdom.
23 However, available evidence supporting fungal VOCs’ roles is mostly weak or circumstantial,

1 and their mechanisms of action remain poorly understood. Additionally, given a very small
2 number of fungi that have been studied for VOC production, many novel VOCs and associated
3 roles likely remain undiscovered. In order to elevate fungal volatiles to the level of other -
4 omes, broad awareness of the significance of fungal VOCs as signals in diverse organismal
5 interactions is critical so that they are not neglected. Because studies on plant-fungal interactions
6 typically involve physical inoculation of fungal cells, even in cases which fungal VOCs function
7 as signals, it would not be possible to recognize them without experiments specifically designed
8 to look for their involvement. For example, growth or stress resistance of the plants exposed to
9 VOCs from PGPF looks similar to what was observed after PGFP inoculation (e.g., Garnica-
10 Vergara et al., 2015, Hung et al., 2013, Naznin et al., 2014), suggesting the likely involvement of
11 VOCs in mediating these benefits. However, research on PGPF has primarily focused on water-
12 borne secreted molecules and their effects on plants and other plant-associated microbes.
13 Because relatively few studies have considered fungal VOCs as the influential signal regulating
14 changes in plant physiology, several critical questions remain mostly unanswered: a) Which
15 fungal VOCs enhance plant growth or stress resistance?; b) Do same VOC(s) affect both traits?
16 If so, how do they manage to increase plant growth while potentially diverting resources to
17 enhance defense?; c) How are such VOCs perceived by plants?; d) Which plant signaling
18 pathways and downstream cellular processes are involved?; and e) How does VOC production
19 contribute to fungal biology and ecology in association with plants?

20
21 Technical challenges involved in VOC analyses are in part responsible for the vast knowledge
22 gaps in the field. One major challenge is the identification of the emitter's intended target(s).
23 Since VOCs can reach a multitude of organisms both in close proximity or relatively distant, and

different organisms may respond differently to the same volatile blend (e.g., Ditengou et al., 2015), it can be difficult to fully understand their ecological purpose and impacts. Especially, negative results are difficult to interpret. Just because one organism does not respond to a fungal volatile blend or individual VOCs does not exclude the possibility of signaling with other organisms within the ecosystem. Some clues can be found in known traits and ecological niches of fungi. For example, obvious potential targets of VOCs from plant-associated fungi are host plants and other organisms commonly associated with the plant (i.e. phytobiome).

Validating putative roles of VOCs also poses significant technical challenges. Application of synthetic version to target organisms is a common method used to determine if a candidate compound elicits similar effects as those caused by a mixture of VOCs from fungal culture (see Table 1 for examples). However, as noted above, concentration is critical as some VOCs can affect an organism differently at different concentrations (e.g., Blom et al., 2011; Splivallo et al., 2007). Therefore, setting up assays for evaluating the effects of individual or combinations of VOCs requires a careful consideration of natural concentrations. Otherwise, some critical functions might be overlooked. Another approach would be to evaluate a target organism's (plant's) response to mutant fungi defective in the production of specific VOCs. This approach was employed to validate multiple roles of bacterial 2,3-butanediol (Cho et al., 2008; Han et al., 2006; Ryu et al., 2003). Bailly et al. (2014) used an *E. coli* mutant that fails to produce indole, in combination with transgenic auxin reporter lines of *A. thaliana* and metabolite analysis, to determine if and how bacterial indole enhances plant growth. Rapid advances in fungal genome sequencing facilitate the identification of biosynthetic pathways for various VOCs, which should help guide the genetic manipulation of their production. Genomes of a large number of fungi that

1 represent diverse ecological niches and phylogenetic lineages, along with their VOC profiles,
2 should also facilitate the identification of candidate biosynthetic pathways and resulting products
3 that may play critical roles in specific biological functions and ecological niches.

4
5 One urgent technical need to facilitate and expand our knowledge on how fungal VOCs affect
6 plants and how resulting changes contribute to fungal ecology in association with plants is the
7 development of more versatile experimental set-up(s). Most studies employed I Petri plates, in
8 which a few plant seedlings and a fungal or bacterial strain were cocultivated in a manner that
9 prevented their physical contact (e.g., Bitas et al., 2015 and Ryu et al., 2003 to see how they
10 were set up). This method has some merits (e.g., easy to set up, quick screening of a large
11 number of fungal isolates), but also has a few shortcomings. One of which is limited space,
12 which makes the assay only suitable for small plants, such as *A. thaliana*, and assays can only be
13 evaluated for a short time period before the plants outgrow the setup. Fast growing fungi and
14 fungi that produce large amounts of air-borne spores tend to contaminate the plant side. This
15 problem can be alleviated by removing a strip of fungal medium along the central divider of I
16 plate (Bitas et al., 2015) and by minimizing plate disruptions. Additionally, this method exposes
17 both the plant roots and shoots to the fungal VOCs and is therefore not suitable for determining
18 which part(s) of plants can perceive candidate compounds. Since most of the bacteria and fungi
19 that have been studied for the production of VOCs affecting plants are soil-borne, a system that
20 limits VOC exposure to roots is highly desirable. An ideal assay system would also allow the
21 observation of diverse plants throughout the entire life cycle, not just the seedling stage, and also
22 allow for volatile fumigations of specific plant organs. The experimental set up used to evaluate
23 how VOCs produced by *Fusarium culmorum*, a cereal pathogen, affect barley growth (Fiers et

1 al., 2013) addressed the issue of specific root volatile fumigation, but is not suitable for long-
2 term observation of plant response. The set up employed by Kai and Piechulla (2009) to study
3 the effect of bacterial VOCs on *A. thaliana* growing in a mixture of sterilized soil/vermiculite is
4 another possibility for evaluating tissue-specificity of VOC perception.

5
6 Another shortcoming of Petri plate-based assays is that plant VOCs may potentially influence the
7 production of fungal VOCs. Since plants also produce a diverse array of VOCs, we cannot
8 overlook the possibility that plant VOCs may affect the fungal physiology in a way that modifies
9 its VOC production. An assay system that allows unidirectional airflow (from fungus to plant
10 and vice versa) is needed. Such a system can facilitate sampling of VOCs for chemical analysis
11 during cocultivation experiments and allow for the manipulation of the amount and types of
12 compounds reaching the other side via the use of material(s) that capture compounds. A system
13 with unidirectional airflow would also help determine if plant growth is affected by elevated
14 levels of CO₂ resulting from microbial respiration (Kai and Piechulla, 2009). When *A. thaliana*
15 was exposed to increasing concentrations of CO₂, ranging from 390 ppm (ambient concentration)
16 to 1680 ppm, fresh shoot biomass stop increasing at concentrations higher than 560 ppm, but the
17 shoot starch content continuously rose with increasing CO₂ concentrations (up to two-fold at
18 1680 ppm) (Van der Kooij et al., 1999). Degrees of plant growth enhancement by VOCs from
19 several soil bacteria grown on nutrient poor media were higher compared with what was
20 observed when they were cultured on nutrient rich media (Blom et al., 2011). Some *F.*
21 *oxysporum* strains failed to enhance plant growth via VOC production, even though their colony
22 was indistinguishable from that of many strains that enhanced plant growth (Bitas et al., 2015).
23 Collectively, these studies suggest that increasing CO₂ due to respiration may contribute to but

1 unlikely be the sole cause of growth enhancement. However, because the concentration of CO₂
2 in the head space of fungal culture flask could reach much higher than 1680 ppm (N. Li,
3 unpublished data), it is important to evaluate potential effect on plant growth by extreme
4 concentrations of CO₂. Addition of a solution of Ba(OH)₂ or KOH, which interacts with CO₂ to
5 form precipitates, in one compartment of Y-plates has been used to reduce the concentration of
6 CO₂ in the head space (Bitas et al., 2015; Franck et al., 2015; Kai and Piechulla, 2009; Lee et al.,
7 2012; Meldau et al., 2013). However, according to our unpublished data, the concentration of
8 CO₂ crashes down initially, but as the concentration of Ba(OH)₂ or KOH decreases over time,
9 the concentration of CO₂ goes up again. Therefore, precise control of the amount of CO₂
10 present in the headspace of plates remains problematic.

11
12 Sampling and subsequent identification of VOCs also present some technical challenges.
13 Among a number of VOC capturing methods (Zhang and Li, 2010), solid phase micro-extraction
14 (Garnica-Vergara et al., 2015; Naznin et al., 2014) and volatile collector traps (Bitas et al., 2015)
15 have been mainly used. Pros and cons of each method were discussed by Zhang and Li (2010).
16 Compounds collected are analyzed via gas chromatography (GC) or GC linked to mass
17 spectrometry (GC-MS) to identify and quantify individual compounds. Identification is
18 performed via a combination of matching retention times on different capillary columns and by
19 both electron impact and chemical ionization MS. Resulting MS spectra are compared with
20 those in reference libraries (e.g., NIST/EPA/NIH Mass Spectral Library) and with spectra of
21 synthetic standards, if commercially available or easy to synthesize, for confirmation of
22 structures. However, certain classes of molecules, such as terpenoids, are underrepresented in
23 such libraries or difficult to synthesize, making their identification challenging. Stereochemistry

determination requires analytical instruments other than MS. Fungi produce quite different VOCs depending on conditions such as nutrients, cultural age, and developmental stage (Bruce et al., 2000; Fiers et al., 2013; Lee et al., 2015; Nemcovic et al., 2008; Wheatley et al., 1997). Therefore, analysis of VOCs captured throughout one short period or cultural condition would likely be inadequate to reveal the full diversity of fungal VOCs and may also mislead during the identification of specific molecules that cause observed effects. Blom et al. (2011) also noted strong influence of culture conditions on bacterial VOC production.

Kingdom Fungi is estimated to consist of millions of species (Blackwell, 2011), many of which perform a diverse array of vital ecosystem services in partnership with plants. However, many plant pathogens and toxin producers also pose a grave threat to global food security and safety. Improved understanding of fungal biology and the mechanism underpinning their interactions with plants and other microbes will facilitate judicious use of beneficial fungi and effective control of pathogens. As such, studies on fungal VOCs that affect plants directly or indirectly will likely help uncover novel mechanisms underpinning plant-fungal interactions and also offer potential applications for improving plant growth and health (Bitas et al., 2013; Kanchiswamy et al., 2015b; Morath et al., 2012). Examples include: a) bio-control/fertilization using bacterial or fungal strains that produce specific VOCs to enhance plant growth and stress resistance; and b) targeted engineering of plant genomes based on an understanding of how they process such VOCs to improve growth and stress resistance. Such strategies are particularly needed to reduce negative environmental impacts from heavy reliance on fertilizers and pesticides for crop production (Rull, 2010; Tilman, 1998). Rapid emergences of resistance to chemicals that control insects, weeds or pathogens have created a boom-and-bust cycle (Lewis et al., 1997),

underscoring an urgent need for alternative approaches. Advances in fungal volatilomics may help address these problems, and therefore this promising frontier warrants further exploration.

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Alcohols

1-Octen-3-ol	2-Methyl-propanol	3-Methyl-butanol	3-octanol	
Aldehydes

2-Ethylhexanal	2-Methylpropanal	3-Methylbutanal	Butanal	Trans-2-octenal
Alkene	Acids		Lactone

Ethylene	Isobutyl acetate	Methacrylic acid	6-Pentyl-α-pyrone	
Benzenoids		Terpenoids

<i>m</i> -Cresol	Methyl benzoate	β-Caryophyllene	(-)-Thujopsene	
Ketones

3-Octanone	Heptan-2-one	1-Octen-3-one	Octan-2-one	Pentan-2-one

Figure 1 Structure of fungal VOCs that control plant growth and/or disease resistance (molecular structure was obtained from ChemSpider.com)

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Table 1 Effects of fungal VOCs on plant growth and disease resistance

Molecule	Fungal species (Strain)	Tested plant(s)	Tested Conc.	References
Fungal VOCs as growth promoters				
β -Caryophyllene	<i>Fusarium oxysporum</i> MSA 35	Lettuce	$\geq 25 \mu\text{M}$,	(Minerdi et al., 2011),
	<i>Talaromyces wortmannii</i> FS2	Komatsuna	134 ppm	(Yamagiwa et al., 2011)
Ethylene	<i>Tuber borchii</i> ,	<i>Cistus incanus</i> ,	Unknown	(Splivallo et al., 2009)
	<i>Tuber melanopsorum</i>	Arabidopsis		
6-Pentyl- α -pyrone	<i>Trichoderma atroviride</i>	Arabidopsis	50-175 μM	(Garnica-Vergara et al., 2015)
(-)-Thujopsene	<i>Laccaria bicolor</i>	Arabidopsis, Poplar	100 ppb	(Franck A. Ditengou et al., 2015)
Mixture	<i>Phoma</i> sp.GS8-3	Tobacco	$1.8 \times 10^{-4} \mu\text{g}$	(Naznin et al., 2013)
Unknown	<i>Alternaria alternata</i>	Arabidopsis, Potato	Unknown	(Ezquer et al., 2010)
Unknown	<i>Cladosporium cladosporioides</i>	Tobacco	Unknown	(Paul and Park, 2013)
Unknown	<i>Fusarium oxysporum</i>	Arabidopsis, Tobacco	Unknown	(Bitas et al., 2015)
Unknown	<i>Trichoderma viride</i>	Arabidopsis	Unknown	(Hung et al., 2013)
Fungal VOCs as growth inhibitors				
2-Ethylhexanal	<i>Trichoderma viride</i>	Arabidopsis	1 ppm	(Hung et al., 2014)
3-Methylbutanal	Many fungi	Arabidopsis	1 ppm	(Hung et al., 2014)
1-Octen-3-ol	Many fungi	Arabidopsis	$\geq 1 \text{ ppm}$,	(Hung et al., 2014),
			130 ppm	(Splivallo et al., 2007)
1-Octen-3-one	<i>Tuber</i> spp.	Arabidopsis	1 ppm	(Hung et al., 2014)
6-Pentyl- α -pyrone	<i>Trichoderma asperellum</i> IsmT5	Arabidopsis	$\geq 2 \text{ mM}$	(Kottb et al., 2015)
Unknown	<i>Cochliobolus sativus</i> ,	Barley	Unknown	(Fiers et al., 2013)
	<i>Fusarium culmorum</i>			
Unknown	<i>Muscodor yucatanensis</i>	Amaranth, Tomato, Barnyard grass	Unknown	(Macías-Rubalcava et al., 2010)
Fungal VOCs as elicitors for ISR				
β -Caryophyllene	<i>Talaromyces wortmannii</i> FS2	Komatsuna	1.34 ppm	(Yamagiwa et al., 2011)
<i>m</i> -Cresol	<i>Ampelomyces</i> spp.	Arabidopsis	$\geq 1 \mu\text{M}$	(Naznin et al., 2014)
Methyl benzoate	<i>Cladosporium</i> spp.	Arabidopsis	$\geq 1 \mu\text{M}$	(Naznin et al., 2014)
3-Octanone	Many bacteria and fungi	Arabidopsis	1 μM	(Naznin et al., 2014)
1-Octen-3-ol	Most fungi	Arabidopsis	0.1 M	(Kishimoto et al., 2007)
6-Pentyl- α -pyrone	<i>Trichoderma asperellum</i> IsmT5	Arabidopsis	$\geq 2 \text{ mM}$	(Kottb et al., 2015)

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