FUNGAL VOLATILE ORGANIC COMPOUNDS AND THEIR EFFECTS ON SEED GERMINATION AND PLANT GROWTH IN ARABIDOPSIS THALIANA

by

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ABSTRACT OF THE DISSERTATION

Fungal Volatile Organic Compounds and Their Effects on Seed Germination and Plant Growth in Arabidopsis thaliana

by Richard Hung

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Dr. Joan W. Bennett

The biological effects of volatile organic compounds (VOCs) have been studied in depth in many organisms. Known volatile interactions include insect–insect or insect–plant interactions, effects of industrial volatiles on humans, plant–plant interactions, plant–bacterial interactions as well as others. Both plant and fungal volatiles have been heavily researched, however, very little research has been conducted to determine the effects of fungal VOCs on plant growth and health.

In order to test these effects in a controlled and reproducible manner, I designed a novel exposure system to assess plant sensitivity to fungal VOCs. This system is designed to expose plants to any microbial or anthropogenic volatile source. In addition, it is scalable to conduct larger experiments and/or expose larger plants to VOCs. With this exposure system, I have determined that Trichoderma viride VOCs induce increased growth in the plant model system Arabidopsis thaliana (45% greater freshweight and 58% greater chlorophyll concentration after four weeks). To determine the volatile or
group of volatiles responsible for inducing growth promotion, gas chromatography and mass spectroscopy were conducted, identifying 56 unique compounds.

After a literature review of volatiles commonly produced by fungi, 23 compounds were selected to test. *A. thaliana* plants were exposed to these compounds individually at 1ppm for 3 days. Various effects on the plants were observed ranging from complete inhibition of seedling formation by 1-octen-3-one to increased fresh weight (4.2%) and chlorophyll concentration (3.7%) in plants exposed to (-)-limonene. No single compound tested induced the growth promotion observed in the *T. viride* exposure experiment indicating that a different compound or a mixture of compounds is possibly responsible for *T. viride* VOC induced growth promotion.

These results show that naturally occurring fungal VOCs have the ability to induce positive or negative changes in plant growth and health. As pressure on agricultural production for fiber, food, and fuel increases for the ever growing human population, volatile gasses will be an important factor to consider as phytostimulants and phytotoxins. The application of stimulatory volatiles for growth enhancement could be used to increase crop yield.
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LITERATURE REVIEW

INTRODUCTION

While human population numbers continue to increase, the amount of land available for agriculture and biofuel production remains constant. One area of rapid increase is the use of plant products to replace petroleum based products that include plastics and biofuels. In order to accommodate the food and bioproduct needs of an ever increasing population, increases of production per acre are necessary.

The eco-friendly, sustainable green movement emphasizes sustainable biologically-based products to replace synthetic and mined chemical additives. The use of microbes in biocontrol and plant growth promotion offers such an ecologically sound approach. Several fungi have been investigated as biocontrol agents, of which members of the genus *Trichoderma*, a common soil fungus, have received the most attention.

Commonly found in soil and root ecosystems, *Trichoderma* species have been extensively studied for their beneficial effects on plant growth including the production of antibiotics and for their ability to compete against other fungi and pathogenic microorganisms (Harman et al. 2004). Several *Trichoderma* species are known to parasitize plant pathogens such as *Fusarium oxysporum*, *Phytophthora capsici*, and *Rhizoctonia solani*. In addition, fungi in the genus *Trichoderma* directly influence the growth of plant structures leading to an increase in plant biomass above ground and adventitious root formation below ground (Windham et al. 1986; Contreras-Cornejo et al. 2009). The mechanisms by which *Trichoderma* simultaneously suppresses plant pathogens and enhances plant growth encompass mycoparasitism, antibiosis,
competition, solubilization and sequestration of inorganic nutrients, and induced resistance.

These properties have lead to its use as a biofertilizer, bioprotectant, and biocontrol agent in agriculture. In these situations, beneficial Trichoderma are employed in a topical and/or fossorial manner. Proximal association of the fungi with the crop confers protection from pathogens and improves biomass production. Trichoderma is often employed in agricultural fields as a seed treatment. Liquid cultures or spores are mixed into the soil and the seeds are sowed into the inoculated soil. Subsequent treatments of the fungi may be applied as a spray over the plants or in the water during irrigation. Considerable research has been conducted to determine how the direct physical contact between Trichoderma and plants results in improved crop yield (Calvet et al. 1993; Höflich et al. 1994; Ousley et al. 1994; Gravel et al. 2007).

As a biocontrol agent, Trichoderma quickly outgrows and outcompetes fungal pathogens (Hansen et al. 2010). This robust growth prevents competing pathogens from colonizing and harming the plant. In addition, many species of Trichoderma are known mycoparasites. Several species including T. atroviride, T. hamatum, and T. harzianum actively seek out and digest other fungi. It is believed that mycoparasitism is facilitated through the production of chitinases, glucans, and glucosidases (Elad et al. 1982; Chérif & Benhamou 1990; Sahai & Manocha 1993; Inbar & Chet 1995; Benhamou & Chet 1996; Haran et al. 1996; Metcalf & Wilson 2001; Viterbo et al. 2001). Moreover, it has been postulated that Trichoderma acts as a bioprotectant through the production of exudates that retard or inhibit the growth of other fungi in the area (Henis et al. 1984; Krupke et al. 2003; Benítez et al. 2004). In addition, T. asperellum has been shown to
activate induced systemic resistance in *Cucumis sativus* (Shoresh et al. 2005). Induction of this response makes plants less susceptible to attack by common plant pathogens such as *Botrytis cinerea* (Korolev et al. 2008).

The majority of the research performed with *Trichoderma* has been conducted on the effects of direct contact of the growing fungus or its liquid exudates with crops. In particular, research on the mechanism of mycoparasitism has focused on the physical interactions between *Trichoderma* and its host. The enzymes and compounds produced by *Trichoderma* degrade and digest its host (Cherif & Benhamou 1990; Elad et al. 1982, Haran et al. 1996; Inbar & Chet 1995; Sahai & Manocha 1993). Intracellular infiltration by *Trichoderma* in plant roots has been implicated in plant growth promotion and protection (Yedidia et al. 1999). These studies have confirmed that *Trichoderma* and its exudates promote plant growth and health. In contrast, the gaseous emanations of *Trichoderma* have received little attention.

Many well known volatile organic compounds (VOCs) of microbiological origin exhibit biological activity. One interesting line of research shows that bacterial volatiles often induce systemic resistance in plants (Ryu et al. 2003, Kishimoto et al. 2007). In particular, plant growth promoting rhizobacteria (PGPR) are known to produce VOCs that have growth promoting properties on *Arabidopsis* (Farag et al. 2006; Zhang et al. 2007). PGPRs also induce systemic resistance in *Arabidopsis* (Ryu et al. 2003; Ryu et al. 2005). On the other hand, while fungi are known to produce a large number of volatile organic compounds, the VOCs produced by fungi have received limited attention in terms of their relationship to plant pathogenesis or growth promotion. Fungi emit cocktails of dozens to hundreds of unique volatile compounds that fall into many
chemical classes including alcohols, aldehydes, acids, ethers, esters, ketones, hydrocarbons, terpenes and sulfur compounds (Korpi et al. 2009). Therefore, an investigation of the volatiles produced by Trichoderma provides fertile ground for developing a new understanding of the mechanisms involved in both the triggering of the mycoparasitism response and of the phenomenon of plant growth promotion. Because Trichoderma is so widespread in soils, and because of its potential for both biocontrol and plant growth promotion is so great, this research has significant implications for improvement of both food and biofuel crops.

The traditional use of Trichoderma as a soil treatment increases the concentration of volatiles generated by Trichoderma in the area, above background concentrations. The concentrations become further elevated in closed growing conditions such as greenhouses, hoop farms, and urban farms. High concentrations of VOCs can have significant effects on plants in the vicinity. One group of volatiles that is of particular interest are eight-carbon volatiles. They are a common product of fungal metabolism produced by nearly all fungi and are responsible for the distinctive odors associated with molds, mushrooms, and certain mold-ripened cheeses. They are products of the oxidation and cleavage of linoleic acid and exist in a range of derivatives, positional isomers and stereochemical variants. Some function as signaling agents in insect attraction and deterrence (Combet et al. 2006) and are particularly associated with the odor of fresh mushrooms (Maga 1981). Compounds in this class include 1-octanol, 3-octenal, 3-octanone, 1-octenol, 2-octenol and 1-octen-3-one. For example, 1-octen-3-ol, sometimes called “mushroom alcohol,” represented 33-78% of the volatile fraction of Agaricus bisporus, 66% of Cantharellus cibarius, 49%-82% in Boletus edulis, and 90%
in *Lactarius torminosus* (Maga 1981). The major C-8 alcohols are available commercially and can be used to test calibrated concentrations of single compounds in controlled microhabitats. In particular, the chiral compound 1-octen-3-ol is one of the most abundant VOCs produced by nearly all species of fungi tested to date. The R enantiomer is said to smell fruity and mushroomy whereas the S enantiomer smells moldy. Different species of mold produce different quantities of the enantiomers (Combet et al. 2006). The production of 1-octen-3-ol is coupled with that of 10-oxo-trans-8-decanoic acid (ODA) as end products of the enzymatic breakdown of linoleic acid though the action of a lipoxygenase (LOX) and a hydroperoxide lyase (Morawicki et al. 2003). At high concentrations (130 ppm), Splivallo et al. (2007) demonstrated that racemic 1-octen-3-ol inhibited root growth and lowered chlorophyll concentration in *Arabidopsis thaliana*. 
GOALS

The overreaching, long term objective of this research is to improve biomass production of food and fuel crops. To this end, this project has four shorter term goals. The first goal is to design a model system using *Arabidopsis thaliana* in which plants can be reproducibly and reliably exposed to controlled environments with different VOCs from molds and synthetic VOCs singly and in mixture. The second goal is to determine the effects of naturally produced mixtures of *Trichoderma* VOCs on *A. thaliana*. The third goal is to determine, through the testing of authentic standards of individual fungal VOCs, possible plant effectors and the positive or negative outcomes of exposure on plant growth. A subgoal of this project is to determine the effects of the different enantiomers of 1-octen-3-ol on seed germination in *A thaliana*. 
RATIONAL AND SIGNIFICANCE

Traditionally, the study of interactions between organisms has been restricted to direct contact either of the organisms themselves or the liquid and solid products of those organisms. Recently published research on plant bacterial interactions have studied the effects of bacterial volatile emissions on plant growth (Ryu et al. 2003). This research has yielded provocative results concerning the growth promoting properties of bacterial VOCs. On the other hand, physical interactions (e.g. mycoparasitism, root colonization) are still the primary emphasis of research in plant fungal interactions with the growth promoting biocontrol fungus *Trichoderma* (Windham 1986; Ousley 1994; Altomare 1999); although it is known that fungi produce a large number of volatiles, many, fungal VOCs such as 1-octen-3-ol are produced in large quantities. I have hypothesized that *Trichoderma* VOCs are important with respect to the biocontrol and plant biomass-enhancing properties of *Trichoderma*. My research has focused on the VOCs produced by fungi, with emphasis on those produced by *Trichoderma*, and their effect on the model plant, *Arabidopsis thaliana*.

In summary, the presence of fungal VOCs can have beneficial, detrimental, or neutral outcomes on plant growth and health. It is important to determine the ability of naturally occurring VOCs to enhance growth rates of agricultural products and keep pace with increasing food and biofuel demands by an increasing population and decreasing fossil fuel reserves.
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Fungal Volatile Organic Compounds: An Overview and Their Role as Ecological Signaling Agents

INTRODUCTION

In terrestrial ecosystems fungi occur as decomposers, symbionts, and pathogens living in close association with bacteria, plants, and animals where inter-organismal signaling is essential. However, the study of interspecific and interkingdom chemical communication has received less scientific scrutiny than signaling within a single organism or signaling within members of a single species.

In this chapter, I provide a general summary of a group of metabolites that we believe have not received sufficient attention by the microbiological community, namely small, easily volatilized molecules that are transmitted in the gas phase. In non-aqueous habitats, volatiles provide a way for microbes to communicate with one another, both within and between species. It is our purpose here to draw attention to the volatile molecules of bacteria and fungi, to document some of the biological versatility that these molecules display, and to highlight their role in fungal associations.

Volatile organic compounds (VOCs) are low molecular weight carbon-containing compounds that evaporate easily at normal temperatures and pressures. The best known volatiles are chemical solvents and other industrial compounds associated with modern life such as paints, cleaning supplies, petroleum fuels, adhesives, photographic solutions, and the like. Exposures to high concentrations of volatiles such as benzene, formaldehyde, methylene chloride, toluene, and xylene are known to have both short- and long-term negative effects on human health (McFee & Zavon 1988).
Far less is understood about biogenic VOCs. Plants and microbes emit a wide range of volatile acids, alkanes, alkenes, carbonyls, alcohols, esters, terpenoids, and other small molecules into the biosphere. The isoprenoids produced by plants are perhaps the best known. In particular, monoterpenes such as limonene ("citrusy"), menthol ("minty"), and pinene ("resinous") have familiar odors. The odors associated with many non-isoprenoid microbial VOCs also are well known -- the earthy smell of garden soil, the noxious stench of spoiled foods, the body odors of people who rarely bathe, and the characteristic mustiness of damp basements are all due largely to gas-phase microbial metabolites. Numerous factors influence the release of VOCs from different biogenic sources including the population of producing species, substrates, temperature, radiation, associations with other organisms, types of ecosystem, and general climate. For comprehensive and provocative reviews on plant VOCs, see Kesselmeier & Staudt (1999) and Baldwin et al. (2006). For a general database of flavors and scents, see Dunkel et al. (2009).

The first major review on fungal volatiles by Hutchinson (1973) focused almost entirely on carbon dioxide. During the 1970s, fungal VOCs were usually isolated by steam distillation followed by liquid--liquid extraction and concentration of the organic extract (Cronin & Ward 1971; Kaminski et al. 1972). These approaches led to the isolation of several major fungal VOCs, but the technical limitations of the methodology gave an underestimate of their number. Since that time, with more sophisticated trapping, separation, and identification techniques, approximately 250 distinct VOCs have been identified from fungi where they occur as mixtures of simple hydrocarbons, heterocycles, alcohols, phenols, thioalcohols, thioesters, and their derivatives (Chiron &
Not all of these VOCs have odors that humans can detect. However, of those that do smell, many of the most familiar ones have extremely low odor thresholds. The human olfactory system is able to detect geosmin, which has an earthy smell, in the range of 150—200 ng/m$^3$. The mushroom-like odor of 1-octen-3-ol is recognized at 10 µg/m$^3$, while the musty odor of 2-octen-1-ol is recognized at 16 µg/m$^3$ (Zogorski et al. 2006).

Complex blends of VOCs cause the distinct bouquets of edible mushroom (Breheret et al. 1997; Tirillini et al. 2000; Cho et al. 2008). For example, the delectable odor of truffles, prized in the culinary arts and said to have aphrodisiacal properties, include over 100 different VOCs of which alcohols, aldehydes, aromatics, and thiols dominate (Splivallo et al. 2007a).

Sulfur-containing volatiles are a highly recognizable group of VOCs. They are best known from plants of the genus Allium (chive, garlic, onion, etc.), where they are valued for their flavors in cooking and for their application in traditional medicine. Similar organosulfur “alliaceous” compounds are made by shiitake mushroom (Lentinula edodes) and species of Tricholoma and Marasmius (Bloch & Deorazio 1994; Sneeden et al. 2004). Sulfur-containing volatiles are an important part of the odor profile of truffles (Mauriello et al. 2004).

Many mushrooms have other distinctive odor profiles. Anise-like odors are characteristic of Clitocybe odora, Lentinellus cochleatus, and Agaricus essettie (Rapior et al. 2002). A “boiled potato” or “farm feed” odor is characteristic of several species of Suillus (de Pinho et al. 2008). The most notorious basidiomycete odor is the repulsive
stench of stinkhorns in the genus *Phallus*, which largely is due to high concentrations of dimethyl oligosulphides (Borg-Karlson et al. 1994). Mushroom smells have been reviewed by de Pinho et al. (2008) and Fraatz & Zorn (2010).

Microscopic fungi similarly emit complex VOC mixtures. Most people are familiar with the musty or moldy odors associated with damp basements and other enclosed indoor spaces. The odor signatures of a given species or strain will vary depending on the substrate, the length of incubation, type of nutrients, temperature, and other environmental parameters. Furthermore, they vary considerably between species (Pasanen et al. 1997; Nilsson et al. 2004; Fiedler et al. 2005). In *Aspergillus* and *Penicillium* species, the concentration of many VOCs increases during sporulation (Börjesson et al. 1993).

The single most commonly reported volatile from fungi, both macroscopic and microscopic, is 1-octen-3-ol, which is also called “mushroom alcohol,” or “matsutake alcohol” because it was first identified from *Tricholoma matsutake* (Wood & Fesler 1986). In the straw mushroom *Volvariella volvacea*, for example, 1-octen-3-ol accounts for the majority (72–83%) of the total volatiles detected (Mau et al. 1997). Mushroom alcohol occurs in two enantiomer forms, \((R)-(\cdot\cdot)\)-1-octen-3-ol and \((S)-(\cdot+)\)-1-octen-3-ol, the \(R\) enantiomer is sometimes called “roctenol.” Roctenol is produced commercially where it is used as an insect attractant (Kline et al. 2007; Bohbot & Dickens 2009). \((R)-(\cdot\cdot)\)-1-Octen-3-ol has a characteristic “mushroomy” odor, while \((S)-(\cdot+)\)-1-octen-3-ol is more grassy or moldy; \((R)-(\cdot\cdot)\)-1-octen-3-ol is the predominant naturally occurring enantiomer of 1-octen-3-ol (Mosandl et al. 1986). The structures of a few representative fungal VOCs are given in Fig 1.
ANALYTICAL PROCESSES FOR EXTRACTION, SEPARATION, IDENTIFICATION, AND QUANTIFICATION

The large number of VOC chemical structures, their generally low concentrations, and the fact that they tend to occur in mixtures pose challenges for comprehensive sampling and analysis. Depending on the situation and intended application, different approaches are utilized for sampling, sample preparation, separation, identification, and quantification. For a good review of current techniques in use for biologically produced VOCs, see Zhang & Li (2010).

Traditionally, an initial step of separation into purer components was followed by identification and quantification. In recent decades, the purging and trapping of headspace gases has gained favor, as for example in studies of odor formation in agricultural settings (Abramson et al. 1980, 1983). The trapping of headspace gases also has been used to obtain VOC profiles of a number of fungal species grown under standardized laboratory conditions (Mattheis & Roberts 1992; Börjesson et al. 1993). The absorbance materials used for trapping (e.g. charcoal, Super Q, Tenax) can select for compounds with particular binding properties. Similarly, the choice of organic solvents (e.g. dichloromethane, hexane) can affect the VOC profile obtained, sometimes yielding insufficient resolution of highly volatile, early-eluting compounds (Kai et al. 2009). Larsen & Frisvad (1995) showed that very different profiles of fungal VOCs are obtained with different collecting methods.

Most determination (separation and identification) of VOCs now relies on gas chromatography--mass spectrometry (GC-MS) because it integrates powerful separation capability and facilitates both quantification and identification. Depending on the
intended purpose, several methods are currently available. Air sampling onto Tenax
desorption tubes followed by thermodesorption allows accurate sampling at one point in
time. In order to determine VOCs over a longer period of time, passive diffusion
monitors onto charcoal adsorbents can be used. Matysik et al. (2009) found that this
method was particularly suitable in epidemiological studies that attempt to correlate
concentrations of specific VOCs and indoor mold exposure. After separation, the
individual constituents of VOC mixtures usually are identified by mass spectrometry.
Comparison of mass spectra with library spectra or determination of chromatographic
retention indices, ideally in conjunction with the parallel determination of authentic
standards, is used to confirm identity (Stoppacher et al. 2010).

The means of delivering the sample to the vacuum chamber for analysis has also
changed and improved over time. Trapping using the solid phase microextraction
(SPME) fiber technique has emerged an efficient and popular method for assessing VOCs
in a variety of contexts (Jeleń 2003). The fiber is exposed to the headspace atmosphere
and, subsequently, the trapped contents are analyzed by GC-MS. SPME has been applied
widely in the flavor and fragrance industry for monitoring freshness, detecting fungal
contamination in stored products and the like (Stoppacher et al. 2010; Zhang & Li 2010).

“Artificial olfaction” is a new area of sensor technology that promises to
revolutionize VOC detection. Electronic noses (sometimes called artificial noses) have
been developed that attempt to mimic mammalian olfactory systems and are finding
increasing applications in healthcare and other biomedical applications. Interestingly, the
diseases caused by certain microbial pathogens are associated with particular odiferous
compounds, e.g. *Pseudomonas* causes a “grape” odor of skin while the sweat of patients
with rubella is said to smell like “freshly plucked feathers” (Pavlou & Turner, 2000). In laboratory experiments, electronic noses were able to distinguish between uncontaminated samples and those contaminated with dry-rot wood decay fungi (Kuske et al. 2005). A comprehensive historical review of electronic nose technology is provided by Wilson & Baietto (2009; 2011).

Finally, it should be noted that dogs can be trained to detect mold growth with 75-94% accuracy (Griffith et al. 2007).
CLASSIFICATION

The anabolic metabolic pathways of bacteria, fungi, and plants are sometimes divided into the dichotomous categories of primary and secondary metabolism. Primary metabolites are essential to the life of the organism and represent the unity of biochemistry. Examples include Krebs cycle intermediates, amino acids, lipids, and nucleic acids (Berg et al. 2007). In contrast, secondary metabolites are the enormous group of diverse natural products not essential to growth, often of extremely unusual chemical structure, which almost always are restricted in taxonomic distribution. Antibiotics such as cephalosporins, hallucinogens such as the ergot alkaloids, and mycotoxins such as the trichothecenes are well known examples of fungal secondary metabolites (Bennett 1983; Bennett & Bentley 1989; Cole & Schweikert 2003). Secondary metabolites are biosynthesized by special pathways (e.g. polyketides, non-ribosomal peptides, and isoprenoids) and constitute the bulk of the field known as natural products chemistry. Advances in genomics facilitate the detection of their signature gene clusters using bioinformatics approaches (Keller et al. 2005).

Due to their low molecular weights and phase dependent appearance, fungal VOCs as a group sometimes are classified as secondary metabolites. However, there are problems with this simplistic categorization. Secondary metabolites usually are made by only a taxonomically limited number of producing species while most VOCs are found across a broad range of producing organisms. For example, production of the toxic secondary metabolite, aflatoxin is restricted to a few species within the genus *Aspergillus*, while production of the volatile 1-octen-3-ol is widespread across many fungi as well as plants and animals (Cole & Schweikert 2003; Chiron & Michelot 2005; Combet et al.)
Secondary metabolites are generally produced by complex metabolic pathways encoded by clusters of linked genes (Zhang et al. 2005). Although less is known about the pathways that produce VOCs, many of them are either metabolic transformation products of lipids, proteins, heterocyclic metabolites, and other components of living tissues, or are degradation end-products (“waste products”) of fungal catabolic pathways.

The common fungal VOC, 1-octen-3-ol, is a good case in point. It comes from linoleic acid (Wurzenberger & Grosch 1984). Although 1-octen-3-ol is sometimes called a secondary metabolite, it is better classified as a lipid degradation product. Both 1-octen-3-ol and a less volatile 10-oxo-trans-8-decenoic acid are produced through the enzymatic oxidation and cleavage of linoleic and linolenic acids (Wurzenberger & Grosch 1984). In Pleurotus pulmonarius, two separate lipoxygenases may be involved in the production of 1-octen-3-ol and 10-oxo-trans-8-decenoic acid (Assaf et al. 1997).

In summary, not all the “small molecules” outside of the central pathways of intermediary metabolism are secondary metabolites. Therefore, in this review, we will not classify fungal VOCs as either primary or secondary metabolites. Instead, we will describe VOCs according to their number of carbons, their ring structures, their substituent group, such as acids, ketones, aldehydes, terpenes, and the like.
OVERVIEW OF FUNGAL VOLATILES

Fungal VOCs are of both theoretical and practical significance within a number of disparate scientific disciplines. They have been studied for their flavor properties, as indicators to detect the presence of fungal growth, as possible contributors to “sick building syndrome,” and as signals for fungal development. Moreover, in recent years, the VOCs from endophytes have emerged as of particular interest because some of them have shown antibiotic activity while others have potential for possible use as fuel compounds or “biodiesel.” All of this research has revealed that fungal VOC profiles are both complex and dynamic: the compounds produced and their abundance vary with the producing species, the age of the fungal colony, abundance of moisture, the type of substrate, the temperature, and other environmental parameters. A few examples from this enormous and scattered literature are given here.

Exploitation of Fungal VOCs: Flavors and as Indicators of Fungal Growth

Mold-ripened cheeses are among the best known food fermentations involving filamentous fungi. Blue cheeses such as Gorgonzola, Roquefort, and Stilton, and white cheeses such as Brie and Camembert, gain their distinctive flavors from methyl ketones and various alcohols produced by fungal metabolism by species of Penicillium (Karahadian et al. 1985; Gallois & Langlois 1990). Fungi are also used in various commercial bioconversion products for making flavor products that can be considered “natural aromas” (Berger et al. 1992; Schreier 1992).

The study of aroma compounds in beers, wines, and other spirits, and their relationship with the perceived flavor of alcoholic beverages represents a huge field that addresses the volatile compounds associated with yeast fermentation. For introductions
to this huge literature, see for example Meilgaard (1975a, b) and Robinson (2006). I will not attempt to cover food and flavor chemistry in this chapter. For an earlier review on mixed bacterial–fungal food fermentations, see Bennett & Feibleman (2001).

VOCs can be used as indirect indicators of the presence of mold growth, even in the absence of visible colonies. Considerable research has gone into developing methods that detect fungal volatiles as an indirect and non-invasive way to indicate the presence of fungal growth in agriculture, water-damaged buildings, and in art conservation.

Fungal contamination of stored foods and feeds is a worldwide problem in agriculture. Fungi decrease the nutritive value of stored foods, spoil them by creating off-flavors, and can produce mycotoxins that render foods poisonous to human beings and other animals that eat them. Compounds such as geosmin, 1-octen-3-ol, 3-octanol, and 3-methy-1-butanol are regularly found in association with stored grains contaminated with fungi (Börjesson et al. 1989, 1993; Mattheis & Roberts 1992). Fungal VOCs have been used to monitor good and bad food quality (Karlshøj et al. 2007). As electronic nose technology improves, it is hoped that volatile compound “mapping” can be used to predict the levels of fungi found in agricultural products and perhaps to identify individual species (Schnürer et al. 1999).

The VOC profiles of common molds grown on building materials have been analyzed in the laboratory (Sunesson et al. 1995, 1996). Mold VOC profiles have also been studied in water-damaged buildings where compounds such as 2-methyl-1-proponol and 3-methyl-1-butanol have been suggested as useful indicators of mold growth (Wilkins et al. 2000; Claeson et al. 2002; Matysik et al. 2008; Korpi et al. 2009). Fungus-like odors can be recognized by humans at concentrations greater than 0.035
µg/m³. Generally, the background microbial VOC concentrations in mold-free buildings are similar to those found in outdoor air ranging from 2.2 to 8.8 µg/m³. VOC levels significantly higher than the background ranges may indicate an increase in the active microbial production possibly associated with adverse health effects (Ström et al. 1994).

In addition, VOC profiles have been used to detect molds grown on objects of art heritage such as tapestries or on the wooden framework behind a painting. Using VOCs, Joblin et al. (2010) developed an index that was used to compare the level of VOCs in the Lascaux caves contaminated with *Fusarium solani* before and after antifungal treatment.

*Non-Specific Building-Related Illnesses*

Many occupants of damp indoor spaces complain of irritation of eyes and mucous membranes, respiratory discomfort, malaise, headaches, gastrointestinal disturbances, and a variety of other symptoms that are often lumped together and called “non-specific building-related illness,” or by the more controversial name of “sick building syndrome.” Many scientists have hypothesized that this spectrum of adverse health is associated with exposure to airborne bacteria and fungi, their aerosolized toxins, or bacterial metabolites such as endotoxins (Thorn 2001; Straus et al. 2003; Burge 2004; IOM 2004; Li & Yang 2004; WHO 2009). Mycotoxins have received most of the attention as the hypothetical cause of the symptoms of sick building syndrome (Jarvis & Miller 2005; Straus 2009). However, VOCs may also contribute to the adverse health consequences associated with damp indoor spaces, especially in cases where there is no visible evidence of mold growth (Walinder et al. 2005; Mølhave 2009). High vapor pressures, low to medium water solubility, and low molecular weights allow both fungal and bacterial VOCs to persist and migrate in the environment, and to diffuse through enclosed wall cavities, air
conditioning filters and vapor barriers. The level of VOCs measured in the indoor air of mold-infested building varies with the ventilation rate, moisture levels, composition of mold population, and the area of the building/room and is constantly changing. One of the highest reported concentrations for a single VOC, 1-octen-3-ol, found in problem buildings is 900 µg/m³ or 0.16 ppm (Morey et al. 1997). Moreover, 1-octen-3-ol has been reported to be one of the major fungal VOCs emitted by various species of fungi (Aspergillus, Cladosporium, Fusarium, Penicillium, Stachybotrys, etc.) regularly found in moldy and water-damaged buildings (Sunesson et al. 1996) or from composting facilities (Fischer et al. 1999). Studies have shown that gas phase 1-octen-3-ol is more toxic to human embryonic stem cells than is toluene (Inamdar et al. 2011). Moreover, when adult Drosophila flies were exposed for one week to low concentrations of chemical standards of 2-octanone (0.5%), 2,5 dimethylfuran (0.5%; DMF), 3-octanol (0.5%), trans-2-octenal (0.5%), and 1-octen-3-ol (0.1%) they exhibited, respectively, 40, 35, 60, 50, and 100% lethality (Inamdar et al. 2010).

Endophytes

Endophytes are microorganisms that live intercellularly within plant tissues without causing any evident negative effects (Bacon & White 2000). They have been found in almost every plant species examined and are likely to play a significant role in plant community structure (Rodriguez et al. 2009). Fungal endophytes have been studied as a source of novel secondary metabolites (Tan & Zou 2001) and also have gained attention as producers of bioactive VOCs. Muscodor albus (“stinky white fungus”) is an endophyte that produces a blend of VOCs that are inhibitory or lethal to a wide range of bacteria and pathogenic fungi. Using GC-MS, Strobel et al. (2001) found that M. albus
produced a mixture of volatile acids, alcohols, esters, ketones, and lipids, which individually had inhibitory but not lethal effects against test species such as *Fusarium solani, Pythium ultimum,* and *Rhizoctonia solani*. When applied collectively, these same VOCs acted synergistically to kill a broad range of plant pathogenic fungi and bacteria (Strobel et al. 2001). Since the original isolation of *M. albus* from cinnamon tree, several other *Muscodor* strains and species that emit antibiotic mixtures of VOCs have been isolated (Atmosukarto et al. 2005; Zhang et al. 2010). This selective antimicrobial effect can be harnessed against undesirable pathogens and has been termed “mycofumigation.” *M. albus* has been used for the biological control of damping off in broccoli seeds grown in greenhouse soilless mix (Mercier & Jiménez 2004; Mercier & Manker 2005). *Oxyporus latemarginatus*, an endophyte isolated from pepper plants, has also shown positive mycofumigation ability against post-harvest decay organisms (Lee et al. 2009).

VOCs from endophytes may have other biotechnological applications. Growing fungi in the presence of *M. albus* has been used as a selection tool to isolate other fungi that produce bioactive volatiles. Species resistant to the VOCs produced by *M. albus* are then screened for the activity of their own VOC profile. Using this method, an endophytic species of *Gliocladium* was isolated from a Patagonian species of *Eucryphia.* This *Gliocladium* species produced a number of VOCs, of which one of the most interesting was 1,3,5,7 cyclooctatetraene or annulene, an unstable and flammable compound used as a rocket propellant during World War II (Stinson et al. 2003). Strobel et al. (2008) coined the term “mycodiesel” when he found that the endophyte *Gliocladium roseum* produced several VOCs normally associated with diesel fuel.
Endophytic fungi in the genus *Ascocoryne* (Griffin et al. 2010), *Phoma* (Strobel et al. 2011), and *Phomopsis* also synthesize VOCs that have fuel potential. *Phomopsis* species isolated from an orchid in Ecuador produces the bicyclic monoterpane sabinene, a monoterpane with a peppery odor first isolated from plants (Singh et al. 2011). Several related monoterpenes are being investigated as possible components of aircraft fuel (Rude & Schirmer 2009).

*Developmental Signals for Fungal Spore Germination and Growth*

A number of biological activities, including chemotropic interactions, growth coordination, and inhibition of the growth of other fungi are mediated by volatile and non-volatile signaling metabolites. For a useful review, see Leeder et al. (2011).

The fungal sensing processes that prevent premature germination are called “self-inhibition” or “auto-inhibition.” These processes detect spore over-crowding and maximize the chances of survival and colony formation once the spore has germinated. High spore concentrations inhibit germination; with lower spore concentrations, germination proceeds. In general, these effects are reversible and do not affect later mycelial growth. Auto-inhibition has been well studied in *Colletotrichum*, where several germination self-inhibitors have been chemically characterized as non-volatile indole compounds and where these compounds are specific for the producing species (Lax et al. 1985; Tsurushina et al. 1995). The cell density-dependent germination inhibitor systems in fungi resemble quorum-sensing systems described for bacteria (see below).

The most abundant VOC produced by fungi, 1-octen-3-ol, functions as a developmental signal for many species. It is produced along with 10-oxo-trans-8-decenoic acid by the enzymatic breakdown of linoleic acid. Both compounds inhibit
mycelial growth of *Penicillium expansum* at low (1.25 mM) concentrations (Okull et al. 2003). In *Penicillium paneum*, dense suspensions of conidia show poor germination; again the auto inhibitor is 1-octen-3-ol (Chitarra et al. 2004). Volatile compound(s) produced by *P. paneum* under high spore-density conditions also inhibit mycelial growth of other fungal species belonging to a variety of genera, suggesting a range of actions beyond auto-inhibition. It has been hypothesized that 1-octen-3-ol interferes, in a reversible manner, with essential metabolic processes involved in swelling and germination of the conidia (Chitarra et al. 2005).

The effect of 1-octen-3-ol is dependent on its concentration and on the stage of fungal colony formation. In dark-grown cultures of *Trichoderma*, very low concentrations of 1-octen-3-ol, 3-octanol, and 3-octanone induced conidiation. However, at the highest concentrations tested for 1-octen-3-ol (500 µM) and 3-octanol and 3-octanone (both 1000 µM), both conidiation and growth were inhibited (Nemcovic et al. 2008). In another study identifying and profiling the naturally occurring volatile metabolites of *T. atroviride*, 1-octen-3-ol and 3-octanone reached their maximum concentrations simultaneously to conidiation (Stoppacher et al. 2010).

Similar effects of C-8 compounds have been observed in *Aspergillus nidulans* by Herrero-Garcia et al. (2011). High conidial densities yielded auto-inhibition of germination, which was associated with 1-octen-3-ol produced in association with aerial hyphae. The inhibition effect was reversible; conidiospores germinated normally after 1-octen-3-ol was removed. The closely related compound 3-octanone enhanced sporulation responses in aerial cultures (Herrero-Garcia et al. 2011). It also is of interest that several C-18 oxylipins derived from linoleic acid (psi factors) can repress conidiation and induce
premature sexual sporulation in *A. nidulans* (Champe et al. 1987; Champe & el-Zayat 1989). Oxylipins are broadly involved in developmental regulation of aspergilli (Tsitsigiannis & Keller 2007) and 10-oxo-trans-8-decenoic acid stimulates mycelia growth, stipe lengthening and the initiation of fruiting in *Agaricus bisporus* (Mau et al. 1992). Oxylipins have been found from fungal pathogens of both plants and animals. While the oxylipin chemical end products are similar, the fungal enzymes involved in their production are different from those isolated from plants and animals. Several lines of evidence suggest that plant oxylipins are able to mimic fungal oxylipins, suggesting a form of “reciprocal cross-talk” in certain host--pathogen relationships (Brodhagen et al. 2008). For a comprehensive review of fungal oxylipins and the enzymes involved in their production, see Brodhun & Feussner (2011).

Various pheromone communication systems are used to coordinate colony formation, fruit body development, and reproduction in higher fungi (Kües & Navarro-González 2009). In the macrofungus *Agaricus bisporus* VOCs produced by the mycelium inhibited primordium formation; 1-octen-3-ol had the greatest inhibitory effect (Noble et al. 2009).

Ammonia is a simple volatile compound not usually thought of as a signaling molecule. Nevertheless, colonies of the yeast *Candida mogii* colonies produce pulses of ammonia that synchronize the development in neighbouring colonies yielding extensive cell and colony morphology changes (Palkova et al. 1997; Palkova & Forstova 2000). Transition to the phase of intense ammonia production (“alkali phase”) is associated with a decrease of mitochondrial oxidative catabolism, peroxisome activation and activation of
biosynthetic pathways that decrease the general stress level in colonies (Palkova et al. 2002).
OVERVIEW OF BACTERIAL VOLATILES

Bacterial species, like fungi, produce complex cocktails of VOCs. For example, a survey of 26 species in the genus *Streptomyces* revealed mixtures of alcohols, alkanes, alkenes, ketones, terpenoids, and thiols in a range of concentrations and combinations (Schöller et al. 2002). Forty-two different volatiles were found from *Myxococcus xanthus* (Dickschat et al. 2005b) and the volatile blend of a marine *Streptomyces* species exhibited antibiotic properties (Dickschat et al. 2005a). For a comprehensive survey of bacterial VOCs, see Schulz & Dickschat (2007).

Bacteria communicate with one another using many molecular mechanisms and they have interactive effects on the organisms with which they share ecological niches including plants, animals, fungi, and other bacteria. Bacterial volatiles have been studied for: (i) their uses in foods, particularly in the dairy industry, (ii) their production of “off” odors in food and water supplies, and (iii) other applied areas. In particular, soil bacteria associated with the rhizosphere have been investigated for their growth-promoting activities. In basic science, the study of quorum sensing has transformed our view of “single-cell” organisms.

*Plant Growth Promotion*

Plant growth promotion by microbial species has been studied in several contexts. Microbes influence plant growth by reducing levels of disease (e.g. by antibiosis or competition with pathogens), stimulating growth, and biofertilization. In some cases, bacteria produce compounds that directly stimulate plant growth. When scientists study these interactions, beneficial effects are usually easiest to demonstrate in the laboratory,
with decreasing success in greenhouses, and only a few microbes functioning successfully in field situations (Lugtenberg & Kamilova 2009).

In addition to secondary metabolites, many growth-promoting rhizobacteria produce volatile organic compounds that have a positive impact on plant growth (Vespermann et al. 2007). Strains of *Bacillus subtilis*, *B. amyloliquefaciens*, and *Enterobacter cloacae* promoted plant growth by releasing several volatiles, of which acetoin and 2,3-butanediol gave the highest level of growth promotion. Mutants of *B. amyloliquefaciens* and *B. subtilis* that are blocked in the biosynthesis of these compounds no longer promoted plant growth (Ryu et al. 2003, 2005).

Long-term exposure to *B. subtilis* volatiles induces beneficial effects on plant growth including increases in plant size and weight, cell numbers, and modulation of root-system architecture (Xie et al. 2009; Gutiérrez-Luna et al. 2010). In addition, there is an increase in the photosynthetic capacity of plants as evidenced by increases in chlorophyll content and up-regulation of genes encoding chloroplast proteins (Zhang et al. 2008; Xie et al. 2009). VOCs stimulate the synthesis of plant hormone-like compounds, including indole-3-acetic acid, cytokinin, and gibberellins. Moreover, genes involved in auxin biosynthesis are up-regulated in plants exposed to VOCs from growth promoting rhizobacteria (Ryu et al. 2003). Zhang et al. (2007) showed that around 600 genes were differentially expressed in *Arabidopsis* seedlings exposed to VOCs from *Bacillus*.

When plant growth-promoting bacteria (PGPR) interact with plants, VOCs are important components of the signaling process (Vespermann et al. 2007). Several bacterial VOCs including alcohols, ammonia, HCN, and phenzine-1-carboxylic acid have
antifungal properties that contribute to the biocontrol properties of PGPR (Whipps 2001; Choudhary et al. 2008; Kai et al. 2009). Kai et al. (2009) showed that volatiles from a given bacterial strain cause different responses on different fungi, i.e. associations of fungi and bacteria interact in different ways. The VOCs of PGPR are associated with induced systemic resistance (Farag et al. 2006). Furthermore, VOCs also lead to an enhancement of aroma compounds in basil (Banchio et al. 2009).

Certain bacterial volatiles promote plant health by inhibiting the growth of fungal pathogens. Negative effects on fungi include the inhibition of both sporulation and spore germination. Positive effects on sclerotial and fruit body development were often observed (Mackie & Wheatley 1999; Fernando et al. 2005; Vespermann et al. 2007).

It is important to note that both Gram-negative and Gram-positive bacteria almost always are studied in the context of aqueous environments. In the laboratory, the approach is to use shaken liquid-batch cultures whereby the quorum-sensing response is detected at a specific point in the growth curve, coinciding with a threshold concentration of signal (Horswill et al. 2007). Because of the dominant aqueous experimental model, it is not surprising that only a few quorum-sensing signals that operate through the gas phase have been discovered. Nevertheless, some cases have been reported. Ralstonia (Pseudomonas) solanacearum is a Gram-negative soil-borne plant pathogen that causes bacterial wilt in a wide variety of important crops including eggplant, potato, and tobacco (Agrios 2008). It uses the volatile signal, 3-hydroxyl palmitic acid methyl ester, for regulating the expression of most of the traits needed for infection and virulence (Cough et al. 1994; Flavier et al. 1997; von Bodman et al. 2003). In environments such as soils and plant surfaces, opportunities for signaling in the liquid phase are limited. Therefore,
it is highly likely that many other volatile auto-inducers will be discovered and that “signaling in the gas phase may be one of the next important frontiers in quorum sensing” (Horswill et al. 2007).

Fungal quorum-sensing molecules also have been identified. The best studied system is *Candida albicans*, a dimorphic fungus, which grows as a commensal on humans in yeast form but functions as an opportunistic pathogen. The yeast–hypha transition is essential for causing disseminated disease. Hyphal development is suppressed by farnesoic acid, which acts as a quorum-sensing molecule (Hogan 2006). There is increasing evidence that quorum sensing is widespread in fungi and that in many of these systems oxylipins such as 1-octen-3-ol play an important role (Kües & Navarro-González 2009).

Specialized terminology tends to be discipline specific and may cause barriers in communication between scientists from different fields. The jargon used to describe the cell density-dependent signaling systems important in fungal spore germination and fruit body development were developed independently from terms used by bacteriologists to describe bacterial quorum-sensing systems (Table 1). Nevertheless, the concepts have many parallels.
VOCS AS ECOLOGICAL SIGNALING AGENTS

With each passing year, there is growing recognition of the extent of chemical communication in the biosphere and the role that volatile chemicals play in biological signaling. Chemical signaling occurs within individual organisms, between individuals of the same species, and also between different species. As a class, chemical signaling molecules sometimes have been termed “infochemicals” or “semiochemicals.” The latter term is used especially by entomologists. Chemical signaling includes both long- and short-range cellular communication. A major distinction is that pheromones mediate intraspecific interactions while allelochemicals mediate interspecific interactions.

Entomologists and chemical ecologists have been at the forefront of studying chemical signaling molecules and have developed an extensive vocabulary for allelochemicals and other semiochemicals (Table 1). Only some of the infochemicals studied to date are found in the gas phase, but the concepts and definitions are useful whatever the physical states of the signaling molecule.

It is important to note that a single chemical can function in more than one type of interaction; the functions are not mutually exclusive (Nordlund & Lewis, 1976). For example, the nearly ubiquitous fungal volatile, 1-octen-3-ol, depending on the context and biological system, is described as a hormone, pheromone, or allelochemical.

Entomology

Entomologists have been pioneers of chemical ecology so it is not surprising that the best known cases of VOC signaling involve arthropods. A complete survey of this topic would require an encyclopedia of its own; only a few examples are given here as an introduction to this fascinating field. The articles cited give access to the extensive
literature describing chemical communication with an emphasis on fungal gas phase molecules.

Many fungal volatiles, particularly eight-carbon alcohols and ketones, act as semiochemicals and function in insect attraction and deterrence (Dowd & Bartelt 1991; Pierce et al. 1991a, b; Nilssen 1998; Ramoni et al. 2001; Ômura et al. 2002; Luntz 2003). In particular, many blood-feeding insects have highly developed olfactory systems whereby volatile semiochemicals enter the antennae and later bind to olfactory receptor neurons (Weeks et al. 2011). C-8 alcohols emitted by decay fungi may serve as trophic signals for conifer feeding bark beetle prey and as by-pass trophic signals for their predators (Zhang & Schlyter 2004). As assayed using a pitfall olfactometer test, cucujin grant beetles showed strong attraction to 1-octen-3-ol (Pierce et al. 1991b).

Many VOCs are involved in arthropod defense. For example, a combination of 1-octen-3-ol and geosmin functioned as defensive allomones in millipedes (Ômura et al. 2002). 1-Octen-3-ol also interrupts the response of beetles to their aggregation hormone (Poland et al. 2009). Most true bugs (Heteropetera) have scent glands in what has been described as “overwhelming chemical fortification” (Aldrich 1988). Moreover, many species secrete attractant pheromones. For example, various true bugs secrete (E)-2-hexanal, hexanal, hexanoic acid, 2-butyl-2-octenal, β-pinene, limonene, and farnesenes as alarm or trail pheromones for defense against their main predators (Aldrich 1988). For those wishing an introduction to the fascinating world of insect defensive secretions, many of which utilize noxious smelling VOCs such as caprylic acid, I recommend Thomas Eisner’s beautifully illustrated book *For Love of Insects* (Eisner 2003).
Fungal odors are known to attract insects that live on fungi or on substrates that are decayed by fungi (Fälldt et al. 1999). In one interesting case in point, female houseflies do not lay their eggs on animal feces that have been colonized by *Fusarium*, *Phoma*, *Rhizopus*, and other fungi. Using chemical standards, six fungal VOCs that consistently inhibit oviposition were determined: dimethyl disulfide, phenylacetaldehyde, 2-penylethanol, citronellal, and norphytone (Lam et al. 2010).

When the chemical composition of insect attractants is known, in some cases effective artificial pheromones can be prepared to trap insect pests. Moreover, “elucidation of chemical messengers for predatory and weed-feeding bugs may lead to pheromonal husbandry of these beneficial insects” (Aldrich 1988).

*Other Associations*

Plants and microbes produce a variety of chemicals that are used in deterring competitors. Humans categorize some of these chemical defenses as antibiotics (if they selectively kill microbes that we wish to see dead) or toxins (if they kill us or our favored plant or animal species). Many of the best known classical secondary metabolites such as alkaloids are thought to have evolved as defense mechanisms. Plants have exquisitely refined chemical defense mechanisms against insect herbivory, including indirect defenses whereby they emit volatiles that attract natural enemies (Howe & Jander 2008).

A survey of 1500 fungal secondary metabolites published between 1993 and 2001 showed that more than half had antibacterial, antifungal, or antitumor activity (Pelaez 2005). The literature on fungal secondary metabolites is extensive and has been reviewed elsewhere (Turner 1971; Turner & Aldridge 1983; Cole & Schweikert 2003). Far less is known about deterrence activity of plant, fungal, and bacterial VOCs. Nevertheless, a
few fungal VOCs are known to have growth inhibitory effects on plants (Splivallo et al. 2007b). When *A. thaliana* is exposed to 1-octen-3-ol, a major fungal VOC, the defense genes that are associated with wounding or ethylene and jasmonic acid signaling are turned on (Kishimoto et al. 2007). Moreover, plants exposed to 1-octen-3-ol are able to inhibit the expansion of the pathogen, *Botrytis cinerea*, on the infected leaves (Kishimoto et al. 2007). In addition, some soil bacteria emit antifungal VOCs (Liu et al. 2008).

Many fungal species engage in symbiotic interactions with plants as mycorrhizae, endophytes, and lichens. In addition, large numbers of fungi are plant pathogens. While fewer fungi cause diseases in animals, the ones that affect humans are becoming increasingly important in modern medicine. In all these interspecific interactions, chemical conversations are taking place, many of them medicated by VOCs. Because volatile compounds can be sensed as a distance they are ideal for serving as intra- and inter-specific chemical signals or “infochemicals.”

Phytopathologists have long recognized the importance of molecular signaling during plant--pathogen interactions. In the rhizosphere, multiple species of bacteria and fungi interact with plant roots and their exudates. Moreover, it is becoming increasingly apparent that non-pathogenic microbes such as nitrogen-fixing bacteria and mycorrhizal fungi that establish beneficial relationships with plants rely on complex signaling networks (Oldroyd & Downie 2004; Harrison 2005). Ortiz-Castro et al. (2009) have reviewed the role of microbial signals in plant growth and development.

Soil remains an underexplored habitat that contains a rich diversity of microbial life forms. The relevance of secondary metabolites and volatiles in soil ecosystems has been reviewed in the monograph edited by Karlovsky (2008). It has been estimated that a
single gram of soil may contain tens of thousands of different fungal, bacterial, archaeal, and protist species. The soil properties such as nutrient and oxygen availability and the physiological state of the microbial population make dynamic and unique soil-specific communities (McNeal & Herbert 2009). Multispecies microbial populations play a key role in sustaining soil microcosms. Communication within rhizosphere bacterial populations, mycelial colonies, and between fungi and bacteria is mediated by signaling molecules, but we are only beginning to learn the specifics of this chemical information exchange. Gas phase molecules are essential components of these chemical conversations. These compounds can diffuse a long way from their point of origin, and they can persist and migrate in soil environments, areas of dense vegetation, and other microhabitats that harbor interacting populations of bacteria and fungi.

Much of the molecular work on biofilm development has been done in the laboratory in single-species biofilms in the context of quorum-sensing research. However, in nature, biofilms are multispecies communities that can harbor several hundred species where various forms of intercellular signaling are being used. By definition, the specialized vocabulary of “autoinducers” is not appropriate in biofilm communities; nevertheless, many of the same signaling molecules and pathways are in use (Kolter & Greenberg 2006).

The factors that influence sporocarp development in the cultivated mushroom *Agaricus bisporus* have been studied widely. Primordium formation depends on: the presence of a “casing layer” containing appropriate bacteria and sufficient ventilation. The air exchange is important because it removes inhibitory VOCs, especially 1-octen-3-ol produced by *A. bisporus*, but also 3-ethyl-1-hexanol produced by the rye on which the
*A. bisporus* spawn is grown. Pseudomonad populations in the casing metabolize 3-ethyl-1-hexanol, thus removing its inhibitory effect (Noble et al. 2009).

In an unusual “Trojan horse” form of bacterial pathogenesis against nematodes, *Bacillus nematocida* B16 lures nematodes by emitting several volatile organic compounds including benzaldehyde and 2-heptanone that are much more attractive to worms than those from ordinary dietary bacteria. Once the bacteria enter the intestine of nematodes, they secrete proteases that preferentially target essential intestinal proteins, leading to nematode death (Niu et al. 2010).

As these tantalizing cases illustrate, the effect of fungal VOCs both in host-pathogen interactions and in non-pathogen ecological contexts is an emerging frontier for future research.

To sum up, VOCs produced by a given fungal species can have multiple effects on other microbes and organisms and can be used for defense, environmental monitoring, and nutrient acquisition (Wheatley et al. 1997; Bruce et al. 2004; Minerdi et al. 2009). I agree with Tarkka & Piechulla (2007) that it is likely that there are many more multifunctional and multiorganismic volatile-based interactions in ecosystems than have been envisioned in the past. As the analytical methods for assaying VOCs improve, we will find increasing evidence of the crucial importance of VOC-mediated cross-talk between species, not only for fungal--bacterial associations, but also for fungal--plant and fungal--animal associations.
SUMMARY

Fungal VOCs have been studied by scientists from a broad range of subdisciplines for both theoretical and applied reasons. As a result, the published information about VOCs exists scattered widely amongst a diverse scientific literature. In general, entomologists do not cross-reference the microbiological literature, nor do food and flavor chemists pay attention to the physiological activities of the compounds they study. This essay has attempted to highlight some of the major ways in which microbial VOCs have been studied to date and to give examples of the ways in which communication in biological systems is mediated chemically. Numerous plant--fungal, insect--fungal, and bacterial--fungal interactions that involve VOC signaling have been discovered. Many more remain to be elucidated. Technological advances with respect to our ability to detect VOCs and our capacity to monitor their physiological effects in plant, animal, and microbial systems will open the door to new views of the complexities underlying chemical signaling across the biosphere.

The scientific community has just begun to eavesdrop on the chemical discourse conducted via gas phase molecules. As we learn more about chemical signaling, it is becoming increasingly apparent that the significance of biogenic VOCs, including fungal VOCs, has been underestimated. It is my hope that this essay will elicit the interest of fungal biologists to ask questions such as:

- How do microbes use VOCs as intraspecific developmental signals and as interspecific signaling cues?
- In what way can we incorporate the insights of the entomological community into the mindset of bacteriologists and mycologists?
- What kinds of technological tools are needed to expand the study of VOCs?
- By what means can VOC research provide a platform for productive interdisciplinary collaborations?
- How many common volatile compounds like ammonia and 1-octen-3-ol serve unsuspected physiological activities?
- Or to put it simply: why do fungi smell?
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Table 1. Technical terms frequently encountered in discussions of chemical signaling compounds.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition and citations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allelochemical</td>
<td>Broadly, a compound that mediates chemical interactions between organisms (Hooper &amp; Pickett 2004). Allelochemicals were originally termed “allelochemics” by Whittaker &amp; Feeny (1971) to describe toxic chemicals made by a plant to defend itself against competing plants.</td>
</tr>
<tr>
<td>Allomone</td>
<td>An allelochemical that only benefits the organism that emits the chemical signal (Hooper &amp; Pickett 2004) “Originally defined as a chemical substance produced or acquired by an organism which, when it contacts an individual of another species in the natural context, evokes in the receiver a behavioral or physiological reaction adaptively favorable to the emitter” (Brown 1968; Nordlund &amp; Lewis 1976).</td>
</tr>
<tr>
<td>Kairomone</td>
<td>A controversial term for an allelochemical which benefits only the receiving organism, but not the producer; i.e. the emitter does not benefit from the interaction (Nordlund &amp; Lewis 1976; Hooper &amp; Pickett 2004).</td>
</tr>
<tr>
<td>Synomone</td>
<td>An allelochemical which benefits both the producing and receiving organisms.</td>
</tr>
<tr>
<td>Autoinducer</td>
<td>A compound used by quorum sensing bacteria to monitor cell density.</td>
</tr>
<tr>
<td>Auto-inhibitor (“self-inhibition”)</td>
<td>A term introduced by Allen (1957) to describe the inhibition of fungal spore germination when spores are overcrowded, a process thought to ensure efficient substrate colonization.</td>
</tr>
<tr>
<td>Crowding effect</td>
<td>The delay and inhibition of spore germination resulting from high spore concentrations (Trinci &amp; Whittaker 1968).</td>
</tr>
<tr>
<td>Growth regulating substances (GRS)</td>
<td>A term sometimes used as a synonym for “plant hormone.” Examples include auxins and gibberellins in plants and possibly 10-oxo-trans-8-decenoic acid in fungi (Takahashi 1986; Mau et al. 1992).</td>
</tr>
<tr>
<td>Chemical ecology</td>
<td>The field that studies how plants, animals, and microbes use chemicals to communicate in natural ecosystems.</td>
</tr>
<tr>
<td>Hormone</td>
<td>A term originally used by endocrinologists to describe chemicals secreted by a gland or group of cells in vertebrates to regulate specific physiological processes within the organism. The definition has been expanded to describe chemicals in other organisms that control and regulate growth and development within the same organism as the compound is secreted.</td>
</tr>
<tr>
<td>Infochemical</td>
<td>Information-conveying chemical: “a chemical that, in the natural context, conveys information in an interaction between two individuals, evoking in the receiver a behavioral or physiological response that is adaptive to either one of the interactants or to both” (Dicke &amp; Sibelis 1988).</td>
</tr>
<tr>
<td><strong>Mushroom alcohol</strong></td>
<td>The common name of 1-octen-3-ol, one of the most prevalent fungal VOCs. Another synonym is matsutake alcohol.</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>PGPB</strong></td>
<td>Plant growth promoting bacteria.</td>
</tr>
<tr>
<td><strong>Psi factors</strong></td>
<td>“Precocious sexual inducers” first identified from <em>A. nidulans</em> by Champe et al. (1987). Champe &amp; el-Zayat (1989) also used the term “sexual sporulation hormone.” These oxylipin compounds are involved in several fungal developmental cascades and are classified according to the fatty acids from which they are derived (Tsitsigiannis &amp; Keller 2007; Brodhun &amp; Feussner 2011).</td>
</tr>
<tr>
<td><strong>Quorum sensing</strong></td>
<td>Ability of bacterial populations to communicate and coordinate their behavior mediated by the use of chemical signaling molecules (Fuqua et al. 1994; Bassler &amp; Losick 2006). Quorum sensing was originally called “autoinduction” (Nealson &amp; Hastings 1979).</td>
</tr>
<tr>
<td><strong>1-Octen-3-ol</strong></td>
<td>An oxylipid C-8 alcohol that is produced by numerous fungi, as well as by many plants, and that also can be emitted by certain arthropods and mammals. It regularly acts as a semiochemical. Depending on the scientific context and literature, it is variously called a pheromone, a kairomone, a synomone or by other jargon terms.</td>
</tr>
<tr>
<td><strong>Oxylipin</strong></td>
<td>A large family of structurally related oxygenated polyenoic fatty acids and the metabolites derived from them. Many oxylipins have physiological activities. They are abundant in mammals but also serve as signals of intra- and inter-cellular communication in other vertebrates, invertebrates, plants and fungi. In microbes, they regulate growth, differentiation, and apoptosis in addition to the development of the infectious processes caused by some pathogenic microorganisms (Noverr &amp; Erb-Downward 2003; La Camera et al. 2004; Tsitsigiannis &amp; Keller 2007).</td>
</tr>
<tr>
<td><strong>Pheromone</strong></td>
<td>An “ecto-hormone”, a chemical signal mediating interactions between organisms of the <em>same</em> species (Hooper &amp; Pickett 2004). A “substance that is secreted by an organism to the outside and causes a specific reaction in a receiving organism of the same species” (Karlson &amp; Luscher 1959; Nordlund &amp; Lewis 1976). Examples include the trail pheromones and sex pheromones that have been well documented in insects.</td>
</tr>
<tr>
<td><strong>Semiochemical</strong></td>
<td>“A chemical involved in the chemical interaction between organisms” (Nordlund &amp; Lewis 1976).</td>
</tr>
</tbody>
</table>
Table 2. Structures, functions and odors of selected common volatile compounds produced by fungi.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Structure</th>
<th>Potential Function(s); Odors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-octen-3-ol</td>
<td><img src="image" alt="Structure" /></td>
<td>semiochemical; earthy, “mushroomy” odor</td>
</tr>
<tr>
<td>1-butanol-3-, methyl-, acetate</td>
<td><img src="image" alt="Structure" /></td>
<td>antifungal; banana odor</td>
</tr>
<tr>
<td>sabinene</td>
<td><img src="image" alt="Structure" /></td>
<td>unknown; peppery odor</td>
</tr>
<tr>
<td>6-pentyl-α-pyrone</td>
<td><img src="image" alt="Structure" /></td>
<td>antibiotic; coconut odor</td>
</tr>
<tr>
<td>β-Caryophyllene</td>
<td><img src="image" alt="Structure" /></td>
<td>plant-growth promoting; woody-spicy odor</td>
</tr>
<tr>
<td>Chemical</td>
<td>Structure</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>isobutyric acid</td>
<td><img src="image" alt="Isobutyric Acid" /></td>
<td>antifungal; rancid cheese-like odor</td>
</tr>
<tr>
<td>benzyl aldehyde</td>
<td><img src="image" alt="Benzyl Aldehyde" /></td>
<td>antimicrobial; almond odor</td>
</tr>
<tr>
<td>1,8-cineole</td>
<td><img src="image" alt="1,8-Cineole" /></td>
<td>antifungal; camphor-like odor</td>
</tr>
<tr>
<td>2-methyl-1-propanol</td>
<td><img src="image" alt="2-Methyl-1-Propanol" /></td>
<td>fungivore attractant; mild alcohol odor</td>
</tr>
<tr>
<td>2-heptanone</td>
<td><img src="image" alt="2-Heptanone" /></td>
<td>unknown; cheese odor</td>
</tr>
<tr>
<td>3-methyl-butanol</td>
<td><img src="image" alt="3-Methyl-Butanol" /></td>
<td>unknown; component of truffle odor</td>
</tr>
</tbody>
</table>
Fig 1. Chemical structures of some representative fungal volatile organic compounds: (A) sabinene, (B) 1-octen-3-ol, (C) geosmin, (D) 3-methyl-1-butanol, (E) 2-methylisoborneal, (F) 3-methyl butyl acetate (isoamyl acetate)
Fig 2. Subdisciplines that have contributed to our knowledge of fungal VOCs.
CHAPTER 1. Arabidopsis thaliana as a Model System for Testing the Effect of Trichoderma Volatile Organic Compounds

INTRODUCTION

Volatile organic compounds (VOCs) are low molecular mass and usually hydrophobic compounds with high vapor pressure, i.e. they easily evaporate at room temperature. They can diffuse a long way from their point of origin and migrate in soil and aerial environments as well as through porous wood materials (Wheatley 2002; Zogorski et al. 2006; Boddy et al. 2008). These physical properties make VOCs useful for interspecies communication as “infochemicals” or “semiochemicals,” especially in non-aqueous environments (Herrmann 2010). Plant VOCs are important in attracting pollinators, warding off herbivores, transmitting signals to neighboring plants and other interspecific communications (Pare & Tomlinson 1999; Pichersky & Gershenzon 2002; Herrmann 2010). Moreover, plant growth promoting rhizobacteria (PGPR) produce specific VOCs that have a positive impact on plant growth both through direct stimulation of plant growth and reductions in the incidence of plant disease (Ryu et al. 2003; Vespermann et al. 2007; Zhang et al. 2008). Other bacterial VOCs have negative effects on plant growth. For example, volatiles from species of Serratia, Pseudomonas and Stenotrophomonas inhibited growth in A. thaliana (Vespermann et al. 2007; Kai et al. 2009, 2010).

Like plants and bacteria, fungi produce a large number of VOCs as mixtures of alcohols, ketones, esters, small alkenes, monoterpenes, sesquiterpenes, and derivatives (Korpi et al. 2009). The kinds, proportions, and concentrations of VOCs vary with the
producing species, its age, the substrate, interactions with other species, and additional environmental conditions (Sunesson et al. 1995; Wheatley et al. 1997; Wilkins et al. 2000). Fungal VOCs have been studied intensively with reference to their use as diagnostic agents for detecting mold growth in agricultural products and in damp indoor environments, their aroma properties in food fermentations, and as fungal-inter kingdom signaling agents (For reviews see Chiron & Michelet 2005; Kues & Navarro-Gonzales 2009; Bennett et al. 2012). They have been implicated as etiological agents in the controversial medical condition called “sick building syndrome” (Mølhave et al. 2009). Further, it is known that mixtures of fungal VOCs produced by several tropical fungal endophytes have both antibacterial and anti-fungal properties (Strobel et al. 2001; Strobel 2006) and VOC-mediated intergenera effects were demonstrated between *Hypholoma fusciculares* and *Resinicium bicolor* (Hynes et al. 2007). Nevertheless, the direct effect of fungal VOCs on plant growth has received relatively little attention. Splivallo et al. (2007) hypothesized that the “burnt” area under trees associated with growth of members of the genus *Tuber* (truffles) was due to VOCs emitted by these subterranean fungi and showed that when *A. thaliana* is exposed to 1-octen-3-ol (“mushroom alcohol”), a major fungal VOC, plant growth was inhibited (Splivallo et al. 2007). Moreover, exposure to 1-octen-3-ol induced expression of the defense genes that are associated with wounding or ethylene and jasmonic acid signaling in *Arabidopsis thaliana* and inhibited growth of the pathogen *Botrytis cinerea* on infected leaves (Kishimoto et al. 2007).

The *Trichoderma* genus has a remarkable range of life styles and displays broad environmental opportunism (Druzhinina et al. 2011). Commonly found in soil and root ecosystems, *Trichoderma* species have been extensively studied for their beneficial
effects on plant growth including the production of antibiotics and ability to compete against other fungi and pathogenic microorganisms (Ouseley et al. 1994; Harman et al. 2004). When *Trichoderma* is inoculated into plants, there is an increase in above-ground plant biomass and below-ground adventitious root formation (Contreras-Cornejo et al. 2009; Windham et al. 1986). Several groups have shown that the VOCs of different *Trichoderma* species can inhibit other fungi, in particular wood decay basidiomycetes and plant pathogens (Dennis & Webster 1970; Wheatley et al. 1997 Humphris et al. 2001; Bruce et al. 2004). In summary, *Trichoderma* has numerous ways of indirectly enhancing plant growth.

The long term goal of this research was to develop *A. thaliana* as a model to study the plant growth effects of natural mixtures of volatiles emitted by biocontrol, plant pathogenic and other fungal groups, thereby expanding upon the research of Splivallo et al. (2007, 2009). The immediate goal of this research was to establish if *Trichoderma viride* VOCs alone, in the absence of direct physical contact, could stimulate plant growth. First, an exposure chamber was developed for exposing *A. thaliana* plants to living cultures of the biocontrol fungus *T. viride* so that the plants and the fungi shared only a common atmosphere. Next, we used several morphological and physiological assays to assess the effect of growing *A. thaliana* in the presence of VOCs from *T. viride*. Finally, the VOC profile of sporulating *T. viride* was determined. To our knowledge, this is the first report of *Trichoderma* VOCs stimulating plant growth in the absence of direct physical contact.
MATERIAL AND METHODS

Fungal and plant growth conditions

*Trichoderma viride* was grown in extra deep (100 x 25 mm) vented Petri dishes on malt extract agar (MEA) (Becton, Dickinson and Company, Product Code 211220). The instructions for this premixed media called for the use of 33.6 g of powder dissolved in 1 L of water. In our experiments, only 25.2 g (75% strength) were dissolved into 1 L of water. Each Petri dish was filled with 60 ml of this media. (When the full strength media was used, the agar surface cracked and dried out before the end of the experiment.) The fungi were grown for one week at 27°C in high humidity before placement in the exposure chamber.

The seeds of *Arabidopsis thaliana* (Ecotype Columbia-7) were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). The seeds were surface-sterilized in a 95% ethanol and 20% bleach solution. The surface-sterilized seeds were sown individually in a 16 x 150 mm test tube containing 10 ml of full strength Murashige and Skoog (MS) with vitamins media (Pytotechnology Laboratories, KS) supplemented with 3% sucrose and 0.03% phytagel (Pytotechnology Laboratories, KS). The test tubes were capped with translucent vented plant tissue culture caps and stratified at 4°C for three days.

The setup for exposure of *A. thaliana* plants to *T. viride* volatiles is illustrated in Figure 1. Forty fully colonized plates of sporulating *T. viride* were placed into a glass chamber with 37.9 L of free volume. The closed Petri dishes were placed in the chamber; the vented lids allowed free gas exchange while preventing the escape of fungal spores. In each trial, one hundred fifty test tubes containing stratified *A. thaliana* seeds were
placed on top of the *T. viride* plates. The exposed plants in test tubes with vented translucent caps were grown in a common atmosphere with *T. viride* at 21°C ± 2°C with a 16 hour photoperiod for 4 weeks. The control plants were put in an identical glass chamber with 40 MEA plates (75% strength) without *T. viride*. At the end of the second, third, and fourth week of exposure to *T. viride* VOCs, 50 plants were removed from both the control and experimental conditions. Each individual plant was weighed and assayed for chlorophyll content. The experiment was repeated three separate times.

**Chlorophyll measurements and data analysis**

The total chlorophyll concentration of individual plants was obtained using the method developed by Jing et al. (2002) with some modifications. The chlorophyll concentration measurements were determined using a spectrophotometer (DU800, Beckman Coulter, Brea, CA) and acetone extracts of whole rosettes. Once the plants had been removed from the test conditions, the roots were severed from the above ground portion of the plant. Shoots and leaves were weighed to obtain a fresh weight. Then chlorophyll was extracted from the above ground portion of each plant using 1 ml of 80% acetone. The plants were soaked overnight at 4°C in darkness prior to obtaining photometric readings at A663 and A645 nm. Each extract contained the chlorophyll from one plant. The total chlorophyll concentration (chlorophyll a and b) was determined with the following equation, \((8.02*A663+20.2*A645)*V/1000*W\), where \(V\) = volume and \(W\) = fresh weight (Palta 1990).

The data were analyzed using Excel software (Microsoft, Redmond, WA) and SigmaPlot (SPSS Science Inc., IL). To test the significance of the exposure studies, Student's t-Tests and ANOVA were performed for each time point (two, three, and four
weeks) comparing controls to exposed plants for both fresh weight and chlorophyll concentration.

**Histochemical Staining**

To stain for the reactive oxygen species (ROS) hydrogen peroxide (H$_2$O$_2$), a modified method developed by Thordal-Christensen et al. (1997) was used. Whole plants were submerged in a 3,3'-diaminobenzidine (DAB) solution (1 mg/ml, pH 3.8) for five hours. Following the staining, chlorophyll was removed by soaking the plant in 95% ethanol overnight.

To detect the presence of endophytic or pathogenic fungi that may have contaminated the cultured *A. thaliana* plants trypan blue staining was used. Trypan blue also stains dead plant cells so its absence denotes plant health (van Wees 2010). Whole plants were submerged in a trypan blue solution (T8154 Sigma-Aldrich Corp., St. Louis, MO) overnight and then chlorophyll was removed by soaking the whole plant in 95% ethanol overnight. Stained plant tissues were examined macroscopically and microscopically.

**Volatile Capture and Analysis**

Total VOC capture and analysis were performed using a purge and trap method. *T. viride* was grown on 75% strength MEA for one week. Controls consisted of 75% strength MEA only. The headspace of the container was purged at 100 mL/min for four hours. The VOCs were adsorbed on six centimeter Tenax columns (Scientific Instrument Services, Ringoes, NJ). The VOCs were recorded and analyzed with a Varian 3400 gas chromatograph (GC) mated to a Finnigan Mat 8230 mass spectrometer (MS). The GC was equipped with a 60 m, Equity-5 (Sigma-Aldrich Corp., St. Louis, MO) column: 0.32
mm diameter, 1 µm film thickness. The VOCs were desorbed onto a -20°C cryotrap with a TD-4 short path thermal desorption apparatus (Scientific Instrument Services, Ringoes, NJ). The GC conditions were as follows: 10:1 split, helium carrier at 20 psi, oven temperature from -20°C to 280°C at 10°C/min. The MS conditions were as follows: positive ion mode, electron impact spectra at 70eV. GC data with standards were used to determine the mass of the compound and the MS of the peaks were determined by their scatter pattern. The compounds found in the MEA control were removed from the data obtained from *T. viride* gas analysis.
RESULTS

After two weeks, plants grown in a shared atmosphere with *T. viride* had larger leaf size and overall root size (Figure 2). Flower bolting and flowering were observed in exposed plants by the end of the third week. By the end of fourth week, the *Trichoderma* exposed plants exhibited a darker hue of green and were clearly larger in size (Figure 3a). In general, *A. thaliana* plants grown in a shared atmosphere with *T. viride* grew more quickly, produced more biomass, and appeared healthier than control plants.

Control and exposed plants had similar root lengths; however, the test plants had greater root fresh weight and more lateral root growth. The average fresh weight of the roots of *Arabidopsis* exposed to *T. viride* VOCs for four weeks was 15.4 mg compared to control plants of 7.2 mg. As seen in Figure 3b, the robust root mass and lateral root growth is easily observed along with the tendency for the growth medium to adhere to the roots when the plant is removed from the culture tubes. This does not occur with control plants.

An increase in plant fresh weight and total chlorophyll concentration was observed in plants exposed to *T. viride* VOCs for 2-4 weeks (Fig 4). At the end of the four week exposure, the average above-ground fresh weight of control plants was 75.1 mg and the average fresh weight of VOC exposed plants was 109.9 mg, a 45% increase (Fig. 4a). The total chlorophyll concentration of *Arabidopsis* was determined at the end of two, three, and four week exposures. At the end of four weeks, the control *Arabidopsis* plants had an average chlorophyll concentration of 5.5 mg/g of fresh tissue. The VOC-exposed *A. thaliana* had an average concentration of 8.8 mg/g (Fig 4b). This represents a 58% increase in total chlorophyll concentration. At two weeks, average
above-ground mass of control plants was 15.0 mg and test plants was 16.8 mg with respective chlorophyll concentrations of 0.4 mg/g and 0.5 mg/g. At three weeks, average above-ground fresh weight was 43.8 mg for control plants and 61.0 mg for test plants with respective chlorophyll concentrations of 2.3 mg/g and 3.7 mg/g.

Student’s t-Tests comparing control and VOC exposed plants were conducted for each collection time (two, three, and four weeks) for both fresh weight and chlorophyll concentration. All Student’s t-Tests resulted in significant values (p < 0.05) indicating statistically significant data sets.

Hydrogen peroxide is one of the first ROS compounds to be expressed when stress pathways are activated in plants. Histochemical staining using DAB was utilized to assay stress responses by measuring the presence of ROS in the tissue. The near lack of DAB staining in leaves of both exposed and control plants indicated an absence of stress responses (Fig 5a).

Trypan blue staining was used to detect the possible presence of fungal hyphae in plant tissues as well as to reveal necrotic plant tissues. Comparison of the exposed and control trypan blue stained leaves showed no differences (Fig 5b) indicating that no endophytic, plant pathogenic or other contaminating fungi were present on the plant throughout the four weeks of *Trichoderma* VOCs exposure and that neither exposed nor control plants were experiencing cell death.

GC-MS analysis was performed on sporulating colonies that had been cultured on MEA for one week. After four hours of collection of headspace, GC-MS analysis revealed 51 unique VOCs (Table 1). Most of the detected compounds were alcohols, ketones, aldehydes or alkenes. The most abundant VOCs detected from *T. viride* under
the growth conditions we used were isobutyl alcohol, isopentyl alcohol, and 3-methylbutanal.
DISCUSSION

We have developed a reliable method of exposing *A. thaliana* plants to living fungal cultures without direct physical interactions between the organisms. The long distance exposure to *T. viride* VOCs resulted in growth promoting effects as evidenced by an increase in fresh weight and root mass, as well as an increase in the concentration of chlorophyll in leaves.

Both fungal and bacterial VOCs are known to have many physiological properties, in particular the ability to inhibit the growth of other microbes. For example, bacteria isolated from canola roots and stubble and from soybean roots, showed antifungal activity in split plate assays. Nonanal, N-decanol, cyclohexanol, ethyl-1-hexanol, benzothiazole and dimethyl trisulfide were identified as the inhibitory volatiles (Fernando et al. 2005). Fungal endophytes in the genus *Mucidor* emit volatile blends with strong antibacterial effects (Strobel et al. 2001; Strobel 2006). When the mixture of VOCs was subdivided, comparable inhibitory effects were not observed, suggesting that several VOCs work synergistically to obtain the antimicrobial activity (Strobel et al. 2001).

Commonly found in soil and root ecosystems, *Trichoderma* species have been extensively studied for their beneficial effects on plant growth including the production of antibiotics and ability to compete against other fungi and pathogenic microorganisms (Harman et al. 2004). These attributes have led to its use as a biofertilizer, bioprotectant, and biocontrol agent in agriculture, especially in countries where farmers cannot afford chemical fertilizers. Several *Trichoderma* species are known to parasitize plant pathogens such as *Fusarium oxysporum*, *Phytophthora capsici*, and *Rhizoctonia solani* (Ahmed et al. 1999; Harman et al. 2004; Sivan & Chet 1989). *Trichoderma* species used
as biocontrol agents grow quickly and aggressively, outcompeting many fungal pathogens and preventing them from colonizing and harming the plant (Carter et al. 1990; Hansen et al. 2010). In addition, many species of *Trichoderma* including *T. atroviride*, *T. hamatum*, and *T. harzianum* are known mycoparasites that actively seek out and digest other fungi through the production of chitinases, glucans, and glucosidases (Cherif & Benhamou 1990; Elad et al. 1982; Haran et al. 1996; Inbar & Chet 1995; Sahai & Manocha 1993). It has been postulated that *Trichoderma* acts as a bioprotectant through the production of exudates that retard or inhibit the growth of other fungi in the area (Benítez et al. 2004; Henis et al. 1984; Krupke et al. 2003). In addition, *Trichoderma asperellum* has been shown to activate induced systemic resistance in *Cucumis sativus* (Shoresh et al. 2005). Induction of this response makes plants less susceptible to attack by common plant pathogens such as *Botrytis cinerea* (Korolev et al. 2008). Finally, the intracellular infiltration of *Trichoderma* into plant roots is also believed to be important in its role in plant growth promotion and protection (Yedidia 1999).

The goal of our research was to develop a reproducible system for studying the effects of *Trichoderma* VOCs on plant growth. We showed that *T. viride* VOCs elicited growth promoting effects in *A. thaliana* in the absence of both direct physical contact and competing plant pathogens. Trypan blue staining indicated that both control and exposed plants were endophyte and pathogen free. We showed that sporulating *T. viride* produces at least 51 VOCs of which isobutyl alcohol, isopentyl alcohol, and 3-methylbutanal were most abundant. No 1-octen-3-ol, a common fungal VOC shown by Splivallo et al. (2007) to inhibit *Arabidopsis* growth, was detected. These naturally emitted VOCs increased plant biomass and chlorophyll concentration in the model system *A. thaliana*. This effect
is similar to that recently observed in lettuce by Minerdi et al. (2009) where the VOCs from a consortium of bacteria and *Fusarium oxysporum* induced a plant growth promotion effect; however, the *Fusarium* VOCs alone did not enhance plant growth.

Rhizobacterial VOCs are known to promote plant growth (Ryu et al. 2003; Zhang et al. 2008). Characterization of the bacterial VOCs and concomitant bioassays demonstrated that compounds 2,3 butanediol and acetoin were the active volatiles (Ryu et al. 2003). Subsequent profiling of volatiles from PGPRs revealed that several branched chain alcohols may also be involved (Farag et al. 2006). In both lettuce and *Arabidopsis*, the bacterial VOC growth effect was associated with the up-regulation of expansin-related proteins in the respective plant species (Minerdi et al. 2011; Zhang et al. 2008). In *Arabidopsis*, lateral root growth and stimulation of biomass are associated with auxin-mediated pathways (Contreras-Cornejo et al. 2009).

To our knowledge this is the first report of the growth promoting effects of *Trichoderma* VOCs without direct physical contact between the fungus and the plants. Contreras-Cornejo et al. (2009) have described *Trichoderma virens*-associated enhancement of biomass and lateral growth development in *Arabidopsis* and hypothesized that auxin-related compounds biosynthesized by *T. virens*, and then diffused through the medium, are responsible for the effect. We suggest that volatile metabolites emitted by *Trichoderma* contribute to the growth-enhancing effect and act as signaling molecules that turn on auxin and/or other plant growth hormone-related pathways. Experiments are underway to identify the specific *Trichoderma* VOCs involved in eliciting the growth promoting effect as well as to identify the specific genetic pathways in the *A. thaliana* plants that are activated by the VOCs. In addition,
future experiments will be performed to determine the efficacy of naturally produced
VOCs by *T. viride* in soil under greenhouse conditions. Direct growth promotion using
VOCs as signaling compounds should be added to the known mechanisms (e.g. antibiotic
production, competition with plant pathogens, defense response elicitation) that
*Trichoderma* employs to enhance plant vigor.
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Farag MA, Ryu C-M, Sumner LW, Pare PW (2006) GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry 67:2262-2268


Shoresh M, Yedidia I, Chet I (2005) Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by Trichoderma asperellum T203. Phytopathol 95:76-84


Table 1.
Headspace volatiles collected from 7 day old *Trichoderma viride*
100 ml/min. Purge Rate, 4 hrs., 1.0 µg Int. Std. by P&T-TD-GC-MS

<table>
<thead>
<tr>
<th>MS Scan #</th>
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<tbody>
<tr>
<td>156</td>
<td>Ethanol</td>
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<tr>
<td>189</td>
<td>Acetone</td>
</tr>
<tr>
<td>285</td>
<td>2-methylpropanal</td>
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<tr>
<td>338</td>
<td>Butanal</td>
</tr>
<tr>
<td>380</td>
<td>isobutyl alcohol</td>
</tr>
<tr>
<td>405</td>
<td>3-methylbutanal</td>
</tr>
<tr>
<td>416</td>
<td>2-methylbutanal</td>
</tr>
<tr>
<td>423</td>
<td>1-butanol</td>
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<tr>
<td>441</td>
<td>2-pentanone</td>
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<td>455</td>
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<tr>
<td>489</td>
<td>Acetoin</td>
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<tr>
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<td>2-methyl-1-butanol</td>
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<tr>
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<td>isopentyl alcohol</td>
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<td>octane</td>
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<td>Hexanal</td>
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<td>Octadiene</td>
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<td>branched nonane isomer</td>
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<td>2-heptanone</td>
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<td>Limonene</td>
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<td>branched C12 paraffin</td>
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<td>805</td>
<td>trans-2-octenal</td>
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<td>octyl alcohol</td>
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<td>898</td>
<td>Decanal</td>
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<tr>
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<td>-----</td>
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<tr>
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<td>201 branched C15 paraffin</td>
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<td>332 geranyl acetone</td>
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<td>Farnescene</td>
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<tr>
<td>1119</td>
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<tr>
<td>1202</td>
<td>222 m.w. sesquiterpene</td>
</tr>
<tr>
<td>1371</td>
<td>pimar-8,15-diene</td>
</tr>
<tr>
<td>1382</td>
<td>kaur-15-ene</td>
</tr>
</tbody>
</table>
Fig 1 - *Trichoderma viride* VOCs exposure chamber setup.
Fig 2 - *Arabidopsis thaliana* exposed to *Trichoderma viride* VOCs for two weeks. The visible indicators of growth promotion in volatile exposed plants were: larger leaves and root mass and increased lateral root development.
Fig 3. *Arabidopsis thaliana* exposed to *Trichoderma viride* VOCs for four weeks. (A) *A. thaliana* exposed to *T. viride* have visible indicators of growth promotion including larger leaves, taller flower stems, formation of several flowers, and earlier flowering. (B) Roots of *A. thaliana*. Plants exposed to *T. viride* have a more robust root mass and increased lateral root development.
Fig 4. *Arabidopsis thaliana* exposed to *Trichoderma viride* VOCs for four weeks. (A) Weekly average fresh weight of above-ground plant mass. (B) Total chlorophyll concentration of above-ground plant mass of *A. thaliana*. The data collected represents the averages of 50 plants per condition repeated 3 times (control and *T. viride* VOCs exposed *A. thaliana*; n=3), per week. Error bars represent the range of the standard error of the mean. Student's t-Tests comparing control and VOC exposed plants within each collection time point (two, three, and four weeks) all resulted in p < 0.05 for both fresh weight and chlorophyll concentration. ANOVA of the fourth week resulted in p < 0.0001 for both chlorophyll and freshweight.
Fig 5. *Arabidopsis thaliana* exposed *Trichoderma viride* VOCs for two weeks. (A) DAB staining of *A. thaliana* leaves. Plants exposed to *T. viride* VOCs and control plants have similar amount of hydrogen peroxide. (B) Trypan blue staining of *A. thaliana* leaves. Both plant leaves look similar.
CHAPTER 2. The Effects of Low Concentrations of the Enantiomers of Mushroom Alcohol (1-octen-3-ol) on Arabidopsis thaliana

INTRODUCTION

Volatile organic compounds (VOC) are organic compounds capable of entering the gas phase under conditions of normal atmospheric temperature and pressure. “Mushroom alcohol,” or 1-octen-3-ol, is an 8 carbon alcohol produced by the enzymatic oxidation and cleavage of linoleic acid (Wurtzenberg & Grosch 1984). It is one of the most abundant volatile organic compounds (VOCs) produced by fungi and is characteristic of fungal aromas and flavors (Mau et al. 1992; Mau et al. 1997; Venkateshwarlu et al. 1999). It has two optically active isomers. The R-(-)-1-octen-3-ol form has a more mushroom like odor while the (S)-(+)1-oct-3-ol form is also mushroom-like with a moldy-grass like note (Mosandl et al. 1986). The compound has a low odor threshold and can be detected by humans at levels of 0.0001 ppm in water (Wnuk et al. 1983). In addition to fungi, this VOC is detected from a widespread group of animals and plants (Bernier et al. 2000; Maggi et al. 2010; Ramoni et al. 2001). In insects, it functions as a signaling molecule (semiochemical), especially in mediating host location cues for flies, mosquitoes and mites where it orients biting flies and other blood sucking arthropods to their hosts (Luntz 2003). Along with CO₂ it is the compound that attracts malaria mosquitoes and can be used as a bait in mosquito traps (Nilssen 1998). In addition to its flavor and arthropod signaling properties, 1-octen-3-ol has been used as an indicator of fungal spoilage in stored grains (Tuma et al. 1989; Schnurer et al. 2002); functions as a self-inhibitor of spore germination in Penicillium paneum (Chitarra et al.
2005); delays the formation of fruiting bodies in *Agaricus bisporus* (Noble et al. 2009); and inhibits the radial growth of microfungi from several genera (Okull et al. 2003; Chitarra et al. 2004). It also has been used to control *Lecanicillium fungicola*, cause of bubble disease in white button mushroom (Berendsen et al. 2013). The literature on the biological activity of 1-octen-3-ol is widely scattered and isolated by discipline; perhaps the most comprehensive single review on its broad range of biological activities is by Combet et al. (2006).

Plant growth promoting rhizobacteria (PGPR) emit a number of VOCs that have beneficial growth effects on their plant hosts (Ryu et al. 2003; Vespermann et al. 2007; Xie et al. 2009; Zhang et al. 2008). Similarly, it has been postulated that biocontrol agents such as *Pseudomonas* and *Trichoderma* may evoke their beneficial effects on plant growth through a variety of mechanisms that may include VOCs (Cook 1993; Howell 2003; Weller 2007; Hung et al. 2013). Nevertheless, compared to bacterial VOCs, far less is known about the effects of fungal VOCs on germination efficiency, seedling formation and plant health.

At high concentrations (130 ppm), Splivallo et al. (2007) demonstrated that 1-octen-3-ol inhibited root growth and lowered chlorophyll concentration in *A. thaliana*. At a low concentration (10 µl of 0.1 M), Kishimoto et al. (2007) showed that 1-octen-3-ol enhanced resistance of mature *A. thaliana* to *Botrytis cinerea* and activated some of the same defense genes turned on by ethylene and jasmonic acid signaling. Both studies employed the racemic version of 1-octen-3-ol and diluted it in commercial solvents. Since chiral discrimination plays a central role in the activity of many biosystems (He & Beesley 2005) and since many commercial solvents have an adverse effect on plant
growth, we have examined the effect of these parameters using *A. thaliana* as our test system. The goal of this research were to determine if low concentrations at 1-3 ppm (as compared to previous published experiments by Splivallo et al. (2007) of the racemic, (S)-(+)\text{-}\text{ocen-3-ol} and R--(\text{-})\text{-}\text{octen-3-ol} forms of mushroom alcohol had different effects on *A. thaliana* seed germination, seedling formation, and plant growth in the absence of commercial solvents.
MATERIAL AND METHODS

Plant material and seed preparation

*Arabidopsis thaliana* seeds (ecotype Columbia 7) were obtained from Dr. Thomas Leustek of the Rutgers University Department of Plant Biology and Pathology, New Brunswick, New Jersey. Surface sterilized seeds were sown on Murashige & Skoog (MS) media with vitamins, 3% sucrose, and 0.3% Gellan Gum Powder (G 434 PhytoTechnology Laboratories, Shawnee Mission, KS). Seeds used in seedling formation studies were sown on Petri dishes with 20 ml MS media, 80 seeds per plate. The sown plates were placed at 4°C in the dark for three days to stratify the seeds. Seeds used to grow plants for exposure assays were sown individually in test tubes with plant tissue culture caps. The test tubes with seeds were then stratified as described above. After three days, stratified seeds were placed in a growth chamber with the following conditions: 21°C ± 2°C and 16 hour photoperiod. After a 72 hour exposure, culture vessels and plants were removed for destructive testing.

Chemicals and exposure conditions

Stratified seeds in Petri dishes were exposed to different concentrations of vaporized 1-octen-3-ol, or to air alone, for a 3 day test period. The racemic form of 1-octen-3-ol was purchased from Sigma-Aldrich (O5284-25G). The enantiomers (S)-(+)1-octen-3-ol and R-(−)1-octen-3-ol were gifts from Bedoukian Research, Danbury, Connecticut. The concentration of each VOC was calculated as mass to volume with one part per million (ppm) defined as 1 µg of compound per liter of free air space. In our germination experiments, three concentration of 1-octen-3-ol were used: 1 ppm, 2 ppm, and 3 ppm. In some preliminary studies, seeds were also exposed to 10 and 100 ppm of
these VOCs. As positive controls, chloroform and dichloromethane were tested at 3ppm. Each Petri plate containing 20 stratified seeds was placed individually into a one liter plant culture vessel with a natural polypropylene closure (PhytoTechnology Laboratories C579). The desired concentrations of VOCs were obtained by the addition by pipette of an aliquot of undiluted liquid compound calculated to deliver the correct concentration. The liquid was deposited on to the surface of the glass on the top third of the vessel along the inside of the vessel. The vessel was sealed to contain the VOC for the duration of the experiment and then placed on a one inch throw rotator at 40 rpm in order to volatilize and evenly distribute the compound, in an incubator at 21°C ± 2°C in the dark. The four vegetative plants were exposed in the same manner as described above for the Petri dishes except for light conditions which was a 16 h photoperiod and VOC concentrations, only 1 ppm was used.

Scoring germination stages

The seeds were exposed for 72 hours and then removed from the culture vessels, examined under a binocular microscope and scored into five germination-to-seedling stages as follows: 1 = no visible germination; seed coat (testa) intact; 2 = initial testa rupture; 3 = radicle emergence (< 1 mm); 4: extended radicle (> 1 mm); 5 = cotyledon visible; complete germination to seeding (see Fig. 1).

Plant mass and chlorophyll concentration

After exposure to 1 ppm 1-octen-3-ol, plants were removed from the test conditions, the roots were removed, and the above ground portion of the plant was weighed to facilitate the analysis of the chlorophyll data which is expressed in relation to the fresh weight of the plant. The excised roots were then weighed independently. The
total chlorophyll concentration of individual plants was obtained using the method developed by Jing et al (2002) with some modifications. The chlorophyll concentration measurements were determined using a spectrophotometer (DU800, Beckman Coulter, Brea, CA) and acetone extracts of whole rosettes. The chlorophyll was extracted using 80% acetone and each solvent extract contained one plant per treatment. The plants were soaked overnight at 4°C in darkness prior to obtaining photometric readings at Absorbance at 663 and 645 nm. The total chlorophyll concentration (chlorophyll a and b) was determined with the following equation, 

$$(8.02 \times A_{663} + 20.2 \times A_{645}) \times \text{Volume}/1000 \times \text{Weight (mass in g)}$$ (Palta 1990).

The data were analyzed and plotted using Excel software (Microsoft, Redmond, WA) and SigmaPlot (SPSS Science Inc., IL). To test the significance of the exposure studies, Student t-tests, and were performed with the aggregated data. There were 100 seeds tested per condition (control, 1, 2, and 3 ppm) in the seedling formation tests. There were 32 vegetative plants tested for each compound at one, two, three, and four weeks of age.
RESULTS

*Exposure concentrations*

In preliminary seedling formation tests at 10 and 100 ppm of 1-octen-3-ol for three days, there were no visible indications of germination, i.e. no seeds reached stage 2. All subsequent tests were done at the lower concentrations. Preliminary tests on exposure of two week old plants at concentrations of 2 ppm and above caused death before the end of the exposure period (three days). All subsequent experiments were done at 1 ppm.

*Germination studies*

Seed germination after exposure of racemic, R- and S- forms of 1-octen-3-ol at 0, 1, 2 and 3 ppm for three days is shown in Figure 2. Between 93-97% of control seeds germinated and formed seedlings (stage 5). Similarly, in solvent controls, at 3 ppm, 95% of seeds exposed to dichloromethane and 94% of seeds exposed to chloroform reached stage 5 (data not shown).

In contrast, seedling formation was retarded at all three levels of exposure to all three forms of 1-octen-3-ol. When seeds were treated with 1 ppm racemic 1-octen-3-ol, only 3% completed germination to seedling; while even fewer (0.25%) seeds reached this stage with exposure to 2 and 3 ppm (Fig. 2a). In general, there was a dose dependent response with increasing retardation of seed germination and seedling formation with higher levels of volatile exposure. With the exception of 1 ppm of the S form, in which 17.4% of seeds reached stage 5; and 1 ppm of the R-form where 5% of seeds reached this stage, levels of retardation of seed germination for each of the two stereoisomers were similar to the racemic form (Fig. 2b, c). No seeds reached stage 5 in the presence of 3
ppm of the R form of 1-octen-3-ol (Fig. 2c). A statistically significant retardation of germination and radicle extension was obtained for 1, 2, and 3 ppm exposure for all three forms of 1-octen-3-ol.

Nevertheless, while seedling formation was retarded, seed germination was not inhibited at these concentrations. At 3 ppm almost half of the seeds had a broken seed coat and over half showed evidence of radicle emergence. Moreover, when removed from the presence of 1-octen-3-ol, the treated seeds completed seedling formation. Experiments at higher concentrations up to 100 ppm showed that treated seeds, although delayed in seedling formation in the presence of 1-octen-3-ol, when removed from the test conditions were able to recover and complete seedling formation at the same frequency as negative controls.

*Vegetative plants*

Exposure of young vegetative plants to 1-octen-3-ol R- and S- enantiomers at 1 ppm caused statistically significant decreases in plant fresh weight at one, two, and three weeks (see Figures 3a-c). Decreased above- and below-ground biomass, were observed. In addition, there was a statistically significant decrease in chlorophyll content at one and two weeks but no differential effect at three and four weeks. There were two instances of chlorophyll concentration increase: the S enantiomer at 1 week and the R enantiomer at 3 weeks. Four week old *A. thaliana* plants exposed to the R enantiomer had an average increase of 0.47mg of chlorophyll content as compared to controls. In both cases where chlorophyll concentration increase was observed, the corresponding fresh weight was decreased, indicating that the chlorophyll concentration increase was due to a smaller plant size and not increased chlorophyll content (see Fig. 3).
In conclusion, the R-form of 1-octen-3-ol is than the S enantiomer in suppressing seedling formation, but in general, both forms of the compound retard, but do not suppress, seed formation. At 1 ppm, both the R- and the S- forms of mushroom alcohol retard growth for one, two, and three week old plants. For three and four week old plants, only the R form has a statistically significant inhibitory effect on chlorophyll content.
DISCUSSION

Plants and their seeds have evolved divergent responses to the environmental signals that involve adaptation to the prevailing environment. In addition to the basic requirements for water, oxygen, and appropriate temperature, plants also may be sensitive to factors such as light, nitrate, and signaling biomolecules. Some VOCs emitted by plants such as allyl isothiocyanate and methyl isothiocyanate play important roles in mediating allelopathic effects and as cues for the presence of proximate competitors (Vaughn & Boydston, 1997; Kegge & Pierik, 2009). These factors interact and affect the ability of seeds to come out of dormancy (defined as the failure of an intact and viable seed to complete germination under favorable conditions (Bewley, 1997)) and of vegetative plants to grow properly.

Many agriculturally and environmentally important chemicals are chiral molecules and sometimes the enantiomers exhibit different biological effects (He & Beesley, 2005). Most of the published literature on 1-octen-3-ol concerns either its properties as a mushroom flavor compound (Zawirska-Wojtasiak, 2004) or its importance in attracting biting insects (Bernier et al, 2000; Luntz, 2003). In both of these cases, the R enantiomer (“roctonal”) is the active component. On the other hand, either of the optically active versions of this alcohol exhibited attracting and molting activities in pine wood nematode (Matsumori et al, 1989), and the racemic form was effective in inhibiting fungal spore germination (Chitarra et al, 2004, 2005; Berendsen et al, 2013). In our studies, the germination of A. thaliana seeds exposed to racemic 1-octen-3-ol and its enantiomers were all retarded. The R form was somewhat more active; however the S form also exhibited significant inhibitory effects, especially on seed germination. I
conclude that chirality is more important to mushroom alcohol’s mechanism of action in arthropod and mammalian olfaction than in spore inhibition in fungi and seed germination inhibition in *A. thaliana*.

It is also important to note that seeds exposed to all three concentrations of 1-octen-3-ol tested were able to resume seedling formation once removed from the testing conditions. This shows that 1-octen-3-ol functions as a retardant, not a toxicant. As plant physiologists learn more about seed dormancy, there is increasing recognition that it is an active physiological state, with complex regulatory networks that integrate environmental signals to regulate germination stages (Finch-Savage & Leubner-Metzger, 2006).

Interestingly, similar effects of mushroom alcohol have been observed in fungi. In *Aspergillus nidulans*, high conidial inoculations yield young colonies that produce 1-octen-3-ol that is associated with “autoinhibition” of germination. The inhibition effect is reversible; conidiospores germinate normally after 1-octen-3-ol was removed (Herrero-Garcia et al, 2011).

In conclusion, at concentrations lower than previously tested, both enantiomers of mushroom alcohol (1-octen-3-ol), a well-known odorant and semiochemical, retard seed germination, seedling formation, and growth in *A. thaliana*, suggesting that other ecologically important aspects of VOC-mediated fungal-plant communications merit further study. *Arabidopsis* mutants affecting different stages of the seed dormancy response and hormone pathways are available, making this plant an excellent model for studying the interkingdom signaling activity of fungal VOCs in general and 1-octen-3-ol in particular.
REFERENCES


Xie X, Zhang H, Pare PW (2009) Sustained growth promotion in Arabidopsis with long-term exposure to the beneficial soil bacterium Bacillus subtilis (GB03). Plant Sig Behav 4:948-953

Table 1. *A. thaliana* seeds exposed to racemic 1-octen-3-ol during seedling formation.

<table>
<thead>
<tr>
<th>Average percent of seeds found in each condition</th>
<th>Control</th>
<th>1 ppm</th>
<th>2 ppm</th>
<th>3 ppm</th>
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<td>1.8</td>
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<tr>
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<td>1.6</td>
<td>5.2</td>
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<td>Stage 3</td>
<td>1.2</td>
<td>13.8</td>
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<tr>
<td>Stage 4</td>
<td>0.8</td>
<td>56</td>
<td>27</td>
<td>12.4</td>
</tr>
<tr>
<td>Stage 5</td>
<td>74.6</td>
<td>2.6</td>
<td>0.4</td>
<td>0.2</td>
</tr>
</tbody>
</table>
1. No germination

2. Germination: Broken Seed Coat

3. Radicle Emergence: < 1mm

4. Extended Radicle: > 1mm

5. Full Seedling formation: Green cotyledons, long root

Fig 1. Stages of seedling formation in *A. thaliana*
Fig 2. Effect of 0, 1, 2 and 3 ppm of 1-octen-3-ol for 3 days on seedling formation in A. thaliana. (a) Average number of seeds that have reached each stage when exposed to racemic 1-octen-3-ol. (b) Average number of seeds that have reached each stage when exposed to R’ 1-octen-3-ol. (c) Average number of seeds that have reached each stage when exposed to S’ 1-octen-3-ol.
Fig 3. Effect of 3 day exposure at 0 and 1 ppm of 1-octen-3-ol enantiomers on 1, 2, 3, and 4 week old *A. thaliana*. (a) comparison of fresh weight (b) comparison of chlorophyll concentration (c) comparison of chlorophyll content per plant. Significance indicated with an asterisk.
CHAPTER 3. Developing *Arabidopsis thaliana* as a Biosensor for Testing the Effect of Fungal Volatile Organic Compounds

INTRODUCTION

Plants are immobile and pervasive. They are an ideal instrument to use to detect harmful organisms such as described by Botanic Gardens Conservation International. They propose the use of plants to detect pests and diseases based on physical infection or attack. However, plants can detect and respond to non-physical signals as well. There is considerable evidence that many soil dwelling bacteria emit volatile organic compounds (VOCs) that are beneficial to plants, for example by inducing systemic resistance (Van Loon et al 1998) and mediating various chemical “conversations” between the rhizosphere and plants (Wenki et al. 2012).

VOCs are low molecular mass compounds with high vapor pressure and low to medium water solubility that exist in the gaseous state at room temperature. Approximately 250 VOCs have been identified from fungi where they occur as complex mixtures of aldehydes, ketones, alcohols, phenols, hydrocarbons, thiols, terpenes, and various derivatives (Chiron and Michelot 2005). Fungal volatiles are the products of both primary and secondary metabolism (Korpi et al. 2009; Turner and Aldridge 1983). VOCs can diffuse through the atmosphere and the soil, making them well adapted for signaling between species that share a common ecological niche, be it natural or artificial.

The gas phase of fungal metabolites (VOCs) have been widely studied for their sensory properties because they impart unique flavors to mold ripened cheeses, Japanese koji and other food products fermented with molds (Steinkraus 1983; Kinderlerer 1989)
and are responsible for the unique bouquet of gourmet mushrooms such as boletes, chanterelles and truffles (Cho et al. 2008; Fraatz and Zorn 2010). Because of their ability to produce off flavors, their presence has been used as an indirect indicator of fungal spoilage in agricultural products (Borjesson et al. 1992; Jelen and Wasowicz 1998; Schnürer et al. 1999). In addition, mycologists have applied them in chemotaxonomic differentiation of mold species (Larsen and Frisvad 1994; Polizzi et al. 2012) and discovered that several fungal VOCs function as inhibitors of spore germination (Tariq and Campbell 1991; Chitarra et al. 2005; Berendsen et al. 2013).

Many plant and microbial volatile molecules function as semiochemicals, otherwise known as “infochemicals.” Both bacterial and fungal VOCs play competitive roles in chemical interactions between microorganisms (Beattie and Torrey 1986), and there is a large literature on the ability of fungal VOCs to mediate arthropod behavior, where they can have properties as pheromones, allomones, and kairomones (Mburu et al. 2011; Rohlfs et al. 2005). Plant growth promoting rhizobacteria may use VOCs to enhance growth in a wide variety of plants (Lugtenberg and Kamilova 2009). The volatiles of non-pathogenic wild-type Fusarium oxysporum (MSA 35) and its bacterial consortium inhibit the growth of a plant pathogenic strain of F. oxysporum (Minerdi et al. 2009). Moreover, lettuce growth was enhanced by the VOCs of strain MSA 35 and its bacterial consortium; the plant growth promotion effect disappeared when the bacteria consortium was removed (Minerdi et al. 2011). In addition, volatile emissions of Bacillus subtilis and B. amyloliquefaciens both stimulated plant growth promotion (Farag et al. 2006).
However, despite the burgeoning literature on inter-kingdom signaling effects of bacterial VOCs on plants and of fungal VOCs on insects, far less is known on the interaction between fungal VOCs and plants (Bitas et al. 2013). I propose the use of plants to detect fungi through volatile interactions. I have determined the physiological effects of fungal volatiles on *Arabidopsis thaliana*. Through literature review, previous and supplemental experiments, 23 common fungal VOCs were chosen to evaluate the effect of these individual volatiles on seed germination, plant growth and chlorophyll content and concentration.
MATERIAL AND METHODS

**GC-MS**

One-week-old *T. viride* grown on malt extract agar (MEA) was placed in a negative pressure glass chamber. Volatiles were collected for analysis according to the method used in Hung et al. 2013 using Super-Q instead of Tenax. VOCs were eluted from the resin column with 1 ml of dichloromethane. The volatiles compounds were analyzed on a HP GC: 10:1 split, helium carrier at 20 psi, oven temperature from -20°C to 280°C at 10°C/min.

**Plant material and seed preparation**

All exposure tests were done with *Arabidopsis thaliana* ecotype Columbia 7. Surface-sterilization of seeds and seedling formation studies were conducted as described previously with slight modifications (Hung et al. 2013). Surface sterilized seeds were sown on Murashige & Skoog (MS) media with vitamins, 3% sucrose, and 0.3% Gellan Gum Powder (G 434 PhytoTechnology Laboratories, Shawnee Mission, KS). Seeds used in germination-seedling formation studies were sown on Petri dishes with 20 ml MS media, 20 seeds per plate. The sown plates were placed at 4°C in the dark for three days to stratify the seeds. Seeds used to grow plants for exposure assays of two–week old plants were sown individually in test tubes with plant tissue culture caps. The test tubes with seeds were then stratified as described above and after three days were placed in the exposure chamber with the following conditions: 21°C ± 2°C and 16 hour photoperiod.

**Chemicals**

The compounds tested, along with their IUPAC names, molecular formulas, and examples of producing fungal species are listed in Table 1. Chemical standards of these
high purity chemicals were purchased from Sigma-Aldrich (St. Louis, Missouri). The 23 compounds used and their purity were: (±)2-methyl-1-butanol (99%), (-)2-methyl-1-butanol (99%), geosmin (97%), isobutyl alcohol (99%), 1-octen-3-ol (98%), octanol (99.5%), 3-octanol (99%), 1-decene (94%), 1-octene (98%), butyraldehyde (99%), 2-ethylhexanal (96%), isobutyraldehyde (99%), isovaleraldehyde (97%), nonanal (95%), 2-heptanone (99%), 2-octanone (98%), 1-octen-3-one (99%), 2-pentanone (99.5%), cyclohexyl isothiocyanate (98%), caprylic acid (99%), (+)limonene (97%), (-)limonene (96%), 2-n-heptylfuran (99%).

Exposure conditions

Both seeds and plants were exposed inside a 1 L glass plant culture vessel with a natural polypropylene closure (PhytoTechnology Laboratories C579). Seeds (20) were exposed in 60 x 15 mm Petri plates. The two-week-old seedlings were placed in individual test tubes, 4 tubes per culture vessel. Before sealing the lids, a 10 x 15 cm piece of Dura Seal Cling Sealing Film (Diversified Biotech.) was placed over the top of each culture vessel as a disposable gasket. The culture vessels containing either seeds in plates or two-week-old plants in test tubes were arranged randomly in the growth chamber. In the case of the vegetative two-week-old plants, three replicates were used in each experiment, and each experiment was repeated three times. Two replicates were used in each seedling formation test, and the experiment was repeated twice.

The concentration of each VOC was calculated as mass to volume with one part per million (ppm) defined as 1 µg of compound per liter of free air space. The individual VOCs were deposited in liquid form. The desired concentration of 1 ppm was obtained by the addition of an aliquot of undiluted liquid compound on to the surface of the inside
of the top third of the glass vessel, calculated to deliver the correct concentration. The vessel was sealed with to contain the VOC for the duration of the experiment and then placed on a 1-inch throw rotator at 40 rpm in order to volatilize and evenly distribute the gas phase compounds. Exposed and control plants were grown for 72 hours in an incubator at 21°C ± 2°C with a 16 h photoperiod.

Scoring germination stages

The seeds were exposed to the individual VOCs for 72 hours and then removed from the culture vessels, examined under a binocular microscope and scored into three stages: no visible germination (testa intact); radicle emergence; seedling formation (cotyledon visible).

Plant mass and chlorophyll concentration

After a 72 hour exposure to individual VOCs, plants were removed for destructive testing. The fresh weight of the aboveground portion of the plants was obtained before measuring the total chlorophyll concentration of the plants using the method developed by Jing et al. (2002), with some modifications. The chlorophyll concentration measurements were determined using acetone extracts of whole rosettes and a spectrophotometer (DU800, Beckman Coulter, Brea, CA). Chlorophyll was extracted using 80% acetone and each solvent extract contained one plant per treatment. The plants were soaked overnight at 4°C in darkness prior to obtaining absorbance readings at 663 and 645 nm. The total chlorophyll concentration (chlorophyll a and b) was determined with the following equation, \( (8.02*A_{663}+20.2*A_{645})*\text{Volume/1000}*\text{W} \) (mass in g) (Palta 1990). The chlorophyll data are expressed both as the total content of an entire plant and concentration per plant.
Statistical analysis

The data were analyzed and plotted using Excel software (Microsoft, Redmond, WA). To test the significance of the exposure studies, one-way analysis of variance (ANOVA) and Student’s t-tests were performed with the aggregated data.
RESULTS

GC-MS

Mass spectroscopy of the volatiles collected with Super-Q from *T. viride* resulted in five compounds: 1-octen-3-one, 1-octen-3-ol, octanone, octanal, and octanoic acid.

Seedling formation tests

Seeds exposed to the volatile compounds geosmin, nonanal, 1-decane, and 2-n-heptylfuran exhibited seedling formation rates similar to that of controls. Seeds exposed to the compounds: 1-octen-3-one, 3-methyl butanal, 2-ethylhexanal, and butyraldehyde were unable to form seedlings. Seeds exposed to the other 15 volatile compounds had intermediate success in seedling formation. Please see Table 2.

Vegetative plants

Plant health of control and VOC-exposed plants were monitored by fresh weight and chlorophyll concentration. Five volatile compounds out of the 24 showed a statistically significant detrimental impact on the fresh weight of *A. thaliana*. The mean fresh weight of plants exposed to 2-octanone, 1-octen-3-ol, 2-n-heptylfuran, limonene, and (-)-2-methyl-1-butanol were, respectively, 8.0 mg, 3.5 mg, 10.8 mg, 24.8 mg and 22.7 mg less than controls.

Five compounds, cyclohexyl isothiocynate, 1-octen-3-ol, 1-decane, 2-heptanone, and (-)-2-methyl-1-butanol caused a statistically significant increase in chlorophyll concentration: 352.2 mg/g, 155.3 mg/g, 139.4 mg/g, 112.9 mg/g, and 109.0 mg/g greater than control, respectively. Three compounds, 2-ethylhexanal, 1-octen-3-one, and geosmin caused a statistically significant decrease in chlorophyll concentration: 394.5 mg/g, 460.9 mg/g, and 195.7 mg/g less than control, respectively. Only two compounds,
1-octen-3-ol and (-)2-methyl-1-butanol, caused statistically significant changes in both fresh weight and chlorophyll concentration. In both cases, the fresh weight was decreased but chlorophyll concentration increased as compared to control.
DISCUSSION

Seedling formation tests showed that although all 23 compounds tested affected seedling formation to some degree, no compound tested caused death at 1 ppm showing that these compounds have an inhibiting but not phytotoxic effect. The experiments exposing two-week-old A. thaliana plants to fungal VOCs also showed a variety of responses. Two compounds, 1-octen-3-ol and 2-heptylfuran caused significant detrimental effect on the fresh weight of plants. However, the chlorophyll concentration of 1-octen-3-ol was not statistically different than the control indicating that although growth was reduced, the photosynthetic capacity of the plant was not adversely affected. On the other hand, the chlorophyll concentration of 2-heptylfuran was statistically higher than that of control. The data from the seedling formation experiments show that 1-octen-3-ol reduced seedling formation to 2.5%, with 72.5% of seeds only able to complete germination and extend the radicle. On the other hand, seeds exposed to 2-heptylfuran completed seedling formation (87.5%) at nearly the same rate as controls (88.8%). Combining the data from the seedling formation experiment and the two-week-old plant exposure tests creates a profile of plant responses to fungal VOCs.

Even in the most meticulously cleaned environment, fungi can grow and flourish. This is particularly true of artificial structures such as houses and offices. Fungi can grow in the walls, in ventilation ducting, beneath the carpet as well as other normally unreachable locations. They can also persist in visible but inaccessible locations such as cracks, crevices, or the underside of furniture and equipment which are present in greenhouses and barns as well. Once entrenched in these locations, the structure
becomes a reservoir of fungi, constantly broadcasting noxious or toxic compounds, which becomes a health concern to the humans and animals in that location.

The purpose of the experiment was to develop *A. thaliana* for use as a fungi biosensor through the different effects of fungal volatiles on plants. As shown in this experiment, as well as others, plants react to fungal VOCs and the responses are varied (Splivallo et al. 2007; Hung et al. 2013; Paul & Park 2013). A catalogue of VOC effects on plants will make it possible to use them as a static low cost biosensor for fungi and VOCs in general.
REFERENCES


Farag MA, Ryu CM, Sumner LW, Pare PA (2006) GC-MS SPME profiling or rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochem 67:2262-2268


<table>
<thead>
<tr>
<th>IUPAC Name (Common Name)</th>
<th>Molecular Formula</th>
<th>Chemical Structure</th>
<th>Example of Producing Species</th>
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<td><strong>Alcohols</strong></td>
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<td>( \text{C}<em>5\text{H}</em>{12}\text{O} )</td>
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<td>Aspergillus versicolor (Sunesson et al. 1995)</td>
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<td></td>
<td>Penicillium commune (Sunesson et al. 1995)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Tuber melanosporum (Li et al. 2012)</td>
</tr>
<tr>
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</tr>
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<td>(Amyl Alcohol)</td>
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<td>Penicillium olsonii (Larsen &amp; Frisvad 1995)</td>
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<tr>
<td></td>
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<td>Tuber melanosporum (Li et al. 2012)</td>
</tr>
<tr>
<td>(4S,4aS,8aR)-4,8a-dimethyl-1,2,3,4,5,6,7,8-octahydropyrenalen-4a-ol (Geosmin)</td>
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<td>Chaetomium globosum (Mattheis and Roberts 1992)</td>
</tr>
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<td></td>
<td></td>
<td>Penicillium expansum (Mattheis and Roberts 1992)</td>
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<td></td>
<td></td>
<td>Penicillium olsonii (Jelen &amp; Wasowicz 1998)</td>
</tr>
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<td>Penicillium expansum (Mattheis and Roberts 1992)</td>
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<td></td>
<td>Penicillium camemberti (Larsen &amp; Frisvad 1995)</td>
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<td>Aspergillus parasiticus (Jelen &amp; Wasowicz 1998)</td>
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<td>Aspergillus ochraceus (Jelen &amp; Wasowicz 1998)</td>
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<td>Fusaria spp (Jelen &amp; Wasowicz 1998)</td>
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<td>Penicillium funiculosum (Jelen &amp; Wasowicz 1998)</td>
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<td><strong>Alkenes</strong></td>
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<td>dec-1-ene (1-Decene)</td>
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<td>Trichoderma viride (Hung et al. 2013)</td>
</tr>
<tr>
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<td>Aspergillus versicolor (Jelen &amp; Wasowicz 1998)</td>
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<td>Penicillium italicum (Larsen &amp; Frisvad 1995)</td>
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<td>butanal</td>
<td>C₄H₈O</td>
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<td><img src="image" alt="Chemical Structure" /></td>
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<tr>
<td>Terpenes</td>
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</table>
| (+)-1-methyl-4-(1-methylethenyl)-cyclohexene (Carvene or (+)-limonene) | C₁₀H₁₆ | ![Chemical Structure](image) | *Emericella nidulans* (Fischer et al. 1999)  
*Penicillium brevicompactum* (Fischer et al. 1999)  
*Penicillium commune* (Jelen & Wasowicz 1998) |
| -1-methyl-4-(1-methylethenyl)-cyclohexene (Achilles diterpene or (-)-limonene) | C₁₀H₁₆ | ![Chemical Structure](image) | *Aspergillus fumigatus* (Fischer et al. 1999)  
*Penicillium commune* (Jelen & Wasowicz 1998)  
*Penicillium crustosum* (Fischer et al. 1999) |
| Furan                    |                   |                    |                             |
| 2-heptylfuran (2-n-Heptylfuran) | C₁₁H₁₈O | ![Chemical Structure](image) | *Trichoderma atroviride* (Polizzi et al. 2012)  
*Trichoderma viride* (Hung et al. 2013) |
Table 2. Seedling formation test arranged from highest average seedling formation rate to lowest.

<table>
<thead>
<tr>
<th>Volatile Compound</th>
<th>Not germinated (%)</th>
<th>Germinated (%)</th>
<th>Formed seedling (%)</th>
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<tr>
<td>1-decene</td>
<td>2.5</td>
<td>8.8</td>
<td>88.8</td>
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<td>2.5</td>
<td>10.0</td>
<td>87.5</td>
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<td><strong>3.3</strong></td>
<td><strong>11.7</strong></td>
<td><strong>85.0</strong></td>
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<td>Geosmin</td>
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<td>20.0</td>
<td>77.9</td>
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<tr>
<td>- limonene</td>
<td>3.8</td>
<td>20.0</td>
<td>76.3</td>
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<td>-2-methyl-1-butanol</td>
<td>5.0</td>
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<td>71.3</td>
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<tr>
<td>+ limonene</td>
<td>3.8</td>
<td>27.5</td>
<td>68.8</td>
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<td>octanoic acid</td>
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<td>66.3</td>
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<td>26.3</td>
<td>65.0</td>
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<td>1-octene</td>
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<td>58.8</td>
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<td>15.0</td>
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<td>78.8</td>
<td>3.8</td>
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<td>butyraldehyde</td>
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<td>83.0</td>
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Fig 1. Effect of 3 day exposure at 0 and 1 ppm of fungal volatiles on two week old A. thaliana. (A) comparison of fresh weight (B) comparison of chlorophyll content per plant (C) comparison of chlorophyll concentration. Significance compared to control is indicated with an asterisk.

<table>
<thead>
<tr>
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</tr>
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<tr>
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</tr>
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CHAPTER 4. Effects of Selected Biogenic and Anthropogenic Volatile Organic Compounds on Arabidopsis thaliana germination and plant growth

INTRODUCTION

Volatile organic compounds (VOCs) are characterized by high vapor pressure, low to medium water solubility, and low molecular weights. They easily become gases or vapors at room temperature and readily migrate in the environment (Hermann 2010). Many municipal, industrial, and agricultural pollutants are volatiles; well known anthropogenic VOCs include benzene, sulfur dioxide, and toluene (Nikolaou et al. 2002; Juang et al. 2010; Zogorski et al. 2006). Industrial VOC emissions encompass many environmental pollutants known for their contributions to the formation of ozone, climate change, and adverse human health effects (Steiner et al. 2006; Ebi & McGregor 2008). These anthropogenic VOCs are commonly emitted from fuels, solvents, paints, adhesives, deodorants, refrigerants, air fresheners, vehicle emissions, fumigants, pesticides and other commonly used products in contemporary society.

Plants and microbes also produce large numbers of VOCs such as hydrocarbons, terpenes, nor-carotenoids, straight-chain alkenes, saturated and unsaturated alcohols, aldehydes, ketones, sulfur derivatives, aromatic compounds, heterocyclics, and many others (Hermann, 2010; Wilkins et al. 2000; Kesselmeier & Staudt 1999; Korpi et al. 2009). Biogenic VOCs are important in affecting atmospheric chemistry (Guenther et al. 1995; Dicke & Loreto 2010) Moreover, many organisms use volatile compounds for signaling and defense, where they variously are called “semiochemicals,” “infochemicals,” “allelochemicals,” or “pheromones.” (Baldwin et al. 2006; Kegge &
Pierik 2010; Bennett et al. 2013). In addition, VOCs have been studied extensively as fragrance and flavor compounds (Berger 2007). Fragrance products containing both synthetic and biogenic VOCs are commonly used as air fresheners and are “generally regarded as safe,” despite the fact that some of these products contain known hazardous compounds and may cause reduction in indoor air quality (Potera 2011).

Most of the research on fungal VOCs has concerned the odor compounds emitted by molds growing in indoor environments and focused either on their use as indicators of mold growth or as possible contributors to “sick building syndrome.” Several groups have developed electronic nose technologies to detect fungal VOCs (Magan 2000; Kuske et al. 2005). It has been hypothesized that fungal VOCs are responsible for at least some of the negative health effects associated with human exposure to damp indoor environmental and accompanying microbial growth (Mølhave 2009; Takigawa et al. 2009). Moreover, it is known that some microbial VOCs have negative effects on rodents, mammalian cell lines, and humans (Kreja & Seidel 2002; Walinder et al. 2005; Korpi et al. 2009). In contrast, several studies have shown that certain microbial volatiles have a positive effect on plant health. Plant growth promoting bacteria (PGPG) produce VOCs that improve plant growth (Ryu et al. 2003; Ortiz-Castro et al. 2009; Bailly & Weisskopf 2012). Minerdi et al. (2011) showed that a mixture of VOCs from a consortium of Fusarium oxysporum and bacteria promote growth in lettuce. In addition, a recent study from our laboratory has shown that an undefined mixture of VOCs from the biocontrol fungus, Trichoderma, enhanced plant size, mass, and chlorophyll concentration in Arabidopsis (Hung et al. 2013). In all of these studies, the growth
stimulation is due to VOC mixtures; the individual bioactive compounds are yet to be determined.

One of the most abundant fungal VOCs, 1-octen-3-ol (“mushroom alcohol”), is a dominant volatile contributing to the odor of cultivated mushrooms and has demonstrable toxic effects in cell culture and on human subjects (Walinder et al. 2005; Korpi et al. 2009). Geosmin, another common microbial VOC, accounts for the characteristic muddy and musty odors associated with soil. It has been shown to function in signaling and communication between microorganisms and has antibiotic activities (Ogura et al. 2000; Watson 2003).

The possible negative effects of microbial VOCs on plant growth and development have received limited research attention. Exposure to several volatiles produced by Tuber species (truffles) inhibited plant growth, induced an oxidative burst in the plant leaf parenchyma tissue, regulated plant root morphogenesis, and increased secondary root formation and hairiness (Splivallo et al. 2007; 2009). Arabidopsis plants treated with 1-octen-3-ol displayed increased resistance against the pathogenic fungus, Botrytis cinerea (Kishimoto et al. 2007). Volatile-mediated death of A. thaliana by bacteria was shown to be caused largely by hydrogen cyanide (Blom et al. 2011).

Previously, my laboratory has shown that natural mixtures of fungal VOC emissions, and chemical standards of individual compounds, can cause toxicity in Drosophila (Inamdar et al. 2010; 2013) and human embryonic stem cells (Inamdar et al. 2011). The objective of this study was to see if fungal VOCs also had physiological effects in plants. I hypothesized that, similar to my observations in Drosophila, plants would show toxic responses to many common volatiles. Therefore, I compared the
effects of low concentrations of several common fungal VOCs, selected commercial solvents (ethanol, formaldehyde, isopropyl alcohol), and some commonly used household fragrance products on seed germination and plant growth in *Arabidopsis thaliana*. 
MATERIAL AND METHODS

Plant Growth Conditions

*Arabidopsis thaliana* seeds (ecotype Columbia-7) were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). The seeds were surface-sterilized using 95% ethanol and 20% bleach solution with 1% Tween 20 solution. For the germination experiments, surface-sterilized seeds were sown onto a 60 x 15 Petri plate containing 10 ml of full strength Murashige and Skoog (MS) medium with vitamins (Pytotechnology Laboratories, KS), 3% sucrose, and 0.03% phytigel (Pytotechnology Laboratories, KS). For vegetative exposure experiments, individual surfaced-sterilized seeds were sown into 25 ml glass test tube containing 10 ml of MS. Vented clear plant tissue culture caps were placed onto test tubes. Surface sterilized seeds were stratified at 4°C for three days and then incubated at 23°C for 14 days prior to exposure to VOCs.

Chemicals

Chemical standards were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and commercial fragrance products were purchased from a local discount retailer (Table 1). The biogenic VOCs were chemical standards of eight VOCs commonly produced by fungi: geosmin, 1-octanol, 2-octanol, 3-octanol, 3-octanone, 1-octen-3-ol, and 1-octen-3-one. The anthropogenic VOCs tested were: ethanol, formaldehyde, isopropyl alcohol and four commercially available fragrance products containing mixtures of VOCs with various delivery mechanisms.

For proprietary reasons, the exact composition of the fragrance products is not available; however, the odor description and hazards identification are given in Table 1.
Concentration of VOCs was defined in parts per million (ppm) of air volume, based on the amount of compound calculated against the internal size of the exposure jar (µL/L).

*Germination and Seedling Formation Exposure to VOCs*

The setup for exposure of *A. thaliana* seeds and vegetative plants to individual VOCs is illustrated in Figure 1. For the germination assay, a closed Petri dish containing 30 surface sterilized *A. thaliana* seeds was placed into a glass tissue culture jar with one liter of free volume. The desired concentrations of VOCs were obtained by the addition by pipette of an aliquot of undiluted liquid compound on to the surface of the glass on the top third of the vessel along the inside of the vessel. One µg of compound was added and the jar was sealed with a translucent polypropylene screw cap. The seeds were exposed to VOCs for 72 hours in a growth chamber at 23±1 °C with a 16 hr photoperiod. The control seeds were exposed to the same conditions without the addition of VOCs. At the end of exposure, the seeds were removed from the test conditions and seed germination and seedling formation efficiencies were examined visually using light microscopy. For the germination and seedling formation assays, the controls were compared to exposed seeds after 72 hrs. The seeds were scored into three categories: no germination, germination (emergence of the radical [embryonic root]), and seedling formation (presence of the radicle, the hypocotyls and the cotyledons) (see Fig. 2). Seeds scored as “no germination” included nonviable seeds and seeds with a broken testa (seed coat) but no radical formation. Three replicates (90 seeds total) were used per treatment and the experiments were repeated four times.

*Vegetative Exposure to VOCs*
Prior to exposure, plants were grown in individual test tubes for 14 days in a growth chamber at 23±1 °C with a 16 hr photoperiod. Four test tubes containing 14-day-old plants were placed into a glass tissue culture jar with one liter of free volume (Fig. 1). One ppm of each VOC was added and the jar was sealed with translucent screw caps. The plants were exposed to the VOCs for the duration of 72 hours in a growth chamber at 23±1 °C with a 16 hr photoperiod. The control plants were placed in identical conditions without any VOCs. At the end of the 72 hour exposure, the plants were removed from experimental conditions and individual plants were weighed and assayed for total chlorophyll concentration. Three replicates were used per treatment and the experiments were repeated three times.

**Plant Chlorophyll Measurements**

Total chlorophyll concentration of plants was determined using the method described by Hung et al. (2013). Individual plants were removed from the test tube, the roots were detached, and the fresh shoot and leaf were weighed. Fresh plant tissue was submerged overnight in 1 ml of 80% acetone in the dark at 4 °C. The resultant chlorophyll extract obtained from a single plant was measured by comparison of the absorption rate at 663 and 645 nm using a spectrophotometer (Beckman Coulter DU800, Brea, CA). The total chlorophyll concentration of chlorophyll a and b was determined from the equation, $$((8.02)(A_{663}) + (20.2)(A_{645}))V/1000W,$$ where V is volume and W is plant fresh weight (Palta 1990).

**Statistical Analysis**

Quantitative results are expressed as means ± standard error of the mean and analyzed using Excel software (Microsoft, Redmond, WA) and SigmaPlot (SPSS Science
Inc., IL). Student’s \( t \)-tests and one-way analysis of variance (ANOVA) between groups were performed for all quantitative data.
RESULTS

Effects of VOCs on Seed Germination and Seedling Development

Seeds were classified into three groups: no germination, germination and progression to seedling formation (Fig. 2). Seeds that have undergone successful germination had protrusion of radicles that varied in length. Seeds that formed into a seedling possessed a radicle with root hairs, hypocotyl, and a pair of green cotyledons.

Percent seed germination and seedling formation at 72 hours are presented in Figure 3. In the absence of VOCs, the germination and seedling growth rates of controls were within the normal range (96%), confirming the natural viability of wild-type seeds. However, the germination rate of seeds exposed to all the tested VOCs differed from controls. Some exhibited signs of embryo expansion and rupturing of testa (seed coat) but without the presence of the radicle. These seeds were classified as seeds having undergone incomplete germination and grouped with seeds that showed ‘no germination’. Approximately 35% of the seeds exposed to 2-octanol and 3-octanone did not germinate. The percentage of non-germinated seeds was greater for 1-octanol (nearly 50%), 1-octen-3-ol (over 60%), and almost complete for 1-octen-3-one (99%). Of those that germinated, seeds exposed to 2-octanol, 3-octanol, and 1-octen-3-ol possessed radicles greater than 2 mm, while seeds exposed to 1-octanol consistently had radicles shorter than 1 mm in length. In the presence of geosmin, another biogenic VOC, we observed 90% seed germination but almost no seedling formation. Of the solvents tested, ethanol, formaldehyde, and isopropyl alcohol were least effective in inhibiting seed germination and seedling formation. Over 75% of the seeds exposed to 1 ppm of ethanol, formaldehyde or isopropyl alcohol progressed to seedling stage and the combined value
of germinated seeds and seedlings were similar to that of controls (92 - 98%). Seeds exposed to the fragrance mixtures had a somewhat higher frequency of incomplete germination compared to seeds exposed to a single anthropogenic compound. Over 70% of the seeds exposed to 1 ppm of the air freshener spray and the stick fragrance oil progressed to seedling stage, as did approximately 50% of those exposed to the scented body mist. “Scented oil of tropical mixed fruit” was the most inhibitory anthropogenic compound. No seeds exposed to the scented oil progressed to seedling formation, and had incomplete germination (58%) and radicle protrusions (42%). Seeds exposed to mixtures of fragrances had an overall decrease in number of seedlings (87 – 92%) and an increase in the number of germinated seeds. In contrast, the seeds exposed to the biogenic VOCs either failed to form seedlings or had a significantly reduced number of seedlings. In summary, the exposure of seeds to any tested VOC at 1 ppm had significant effects on seed germination and seedling development compared to controls (ANOVA, \( P = 0.0001 \)).

At the end of the exposure period, all seeds that were unable to complete seedling formation were removed from the treatment conditions, placed into a clean sterile plant media and allowed to grow for three additional days. Subsequent recovery and seedling formation were 95% (data not shown).

**Effects of VOCs on Plant Growth and Development**

Fourteen-day-old, vegetative *Arabidopsis* plants were exposed for 72 hours to the 14 VOC conditions. After the exposure period, control plants looked healthy with fully expanded green leaves. With the exception of formaldehyde, plants exposed to common anthropogenic compounds singly or air freshener products were all smaller in size but did
not show additional signs of stress such as visible discoloration (Fig. 4A). Formaldehyde-exposed plants had notably reduced root size, were entirely bleached (severe reduction in chlorophyll, causing the plants to look pale yellow to white) and did not resume growth after removal from the exposure conditions (Fig. 4A).

Plants exposed to chemical standards of geosmin and C-8 hydrocarbons exhibited a wider range of symptoms compared to ethanol, isopropyl alcohol and the air freshener products. In addition to being smaller in size, many of these test plants had leaves that curled in and altered root sizes. Plants exposed to 2-octanol exhibited localized cell death where large sections of the leaves were discolored (yellow) or dead (white). For 3-octanone and geosmin, the damage was limited to the smaller leaves (Fig. 4B). Plants exposed to 1 ppm of 1-octen-3-ol were lighter in color with smaller root size, and had small necrotic lesions distributed on most parts of the leaves (Fig. 4C). The most phytotoxic biogenic compound, 1-octen-3-one, caused bleaching and death in plants by the end of the experiments (Fig. 4B).

Effects of VOCs on Plant Chlorophyll

In order to quantify the bleaching observed in VOC-exposed plants, we measured total chlorophyll concentration (Fig. 5). The average chlorophyll concentration of control plants was 1.9±0.1 mg per gram of fresh tissue. The average chlorophyll concentration in plants exposed to common biogenic VOCs, ranged from 0.9 to 1±0.3 mg/g, a decrease of 47 to 53%. The chlorophyll concentration of plants exposed to 1-octen-3-one was the lowest (0.09 ± 0.05 mg/g, a 95% reduction). Plants exposed to ethanol, isopropanol, formaldehyde and the four fragrance products also had reduction in chlorophyll concentration; however, the reduction of chlorophyll in plants exposed to fragrance
mixtures were less in comparison to the biogenic VOCs, with averages ranging from 1.12 to 1.3±0.1 mg/g (32 and 41% decrease, respectively). Of the anthropogenic compounds tested, formaldehyde treatment resulted in the lowest chlorophyll concentration of 0.5±0.08 mg/g (74% decrease). Student’s t-tests comparing control and VOC exposed plants were conducted for each treatment for the total chlorophyll concentration. With the exception of ethanol, the data collected from the treated plants were significantly different from controls (ANOVA, $P = 0.0001$).
DISCUSSION

In this study, I determined the sensitivity of seeds and young plants to the presence of seven biogenic and seven anthropogenic VOCs. The phytotoxicity of the fourteen VOC treatments (72 hours at 1 ppm) was evaluated using seed germination and plant growth assays.

The process of germination begins with the uptake of water by the seed (imbibition), followed by embryo expansion leading to rupturing of the testa. Germination is completed with the rupture of the seed covering layers and emergence of the radicle. Seedling vigor is assessed by evaluating the emergence of radicle, the growth of the radicle, the emergence of hypocotyl and the cotyledons (Rajjou et al. 2012). During germination, the embryonic plant draws its energy from the stored materials within the seed; hence, the embryo is isolated from external hazards and is less affected by external factors in the environment. I made the distinction between the completion of seed germination and seedling formation because seed germination alone has been regarded as a less sensitive method of evaluating phytotoxicity (Araujo & Monteiro 2005).

*Arabidopsis* seeds responded differently to several biogenic C-8 compounds that were similar in molecular size and masses. Exposure to 1-octen-3-one caused complete inhibition of germination while 1-octanol caused radicles to stop growing as soon as they emerged. Other C-8 compounds took longer to impede radicle root growth. The antagonistic activities of ketones, alcohols and aldehydes on seed germination and seedling formation have been demonstrated previously. For example, Vokou et al. (2003) showed that out of 47 terpenoid compounds tested, 24 inhibited seedling
formation in lettuce. Bradow (1991) showed that C-7 and C-8 VOCs from plant residues were inhibitory to germination of carrot, onion and tomato seeds. On the other hand, Splivallo et al. (2007) did not observe significant effects of 1.3 ppm of 3-octanol, 3-octanone, trans-2-octenol or several other volatiles emitted by truffles on primary root or cotyledon formation in A. thaliana. In my studies, 1-octen-3-one (a ketone) was the most effective against seed germination and seedling development. Nevertheless, the inhibitory effects we observed were not lethal. Once removed from the presence of 1-octen-3-one, seeds germinated and grew into seedlings.

Geosmin is a well-known biogenic volatile with an extremely low odor threshold in humans. It is the main source of the distinctive odor associated with soils (geosmin means “earth odor”) and sometimes causes musty odor problems in municipal water supply systems (Jiang et al. 2007). To my knowledge, the only other study of the effect of geosmin on seed germination was conducted by Ogura et al. (2000) on seeds from 15 economically important members of the Brassicaceae (crucifer family), the same group in which A. thaliana is classified. They observed inhibition of seed germination but not seedling formation for several varieties of radish, while little inhibition of germination was observed for cabbage, rape and mustard seeds. In my studies of A. thaliana, geosmin-exposed seeds germinated at almost the same rate as controls (90%) but seedling formation was inhibited. Ogura et al. (2000) were able to restore germination of geosmin-inhibited radish seeds by treatment with gibberellin A. In my studies, once removed from the VOC testing conditions and placed into a fresh plant media, treated A. thaliana seeds were able to complete germination and/or resume vegetative growth without application of a plant hormone.
The results from the vegetative plants exposed to 14 volatile treatment conditions suggest general toxicity of the VOCs we tested. Vegetative plants exposed to ethanol, isopropyl alcohol and the air freshener products were all smaller in size. A greater range of adverse effects were detected in plants exposed to the biogenic VOCs. Observed morphological changes included smaller leaf size, decreased root growth, leaf curling, reduction in the intensity of plant leaf pigment and concomitant reduction in chlorophyll concentration. Roots, which are responsible for the absorption and accumulation of chemicals, are sensitive to environmental signals (Ortiz-Castro et al. 2009; Gutiérrez-Luna et al. 2010).

Exposure to formaldehyde vapors killed plants in less than three days. Lesions on bean plant leaves at concentrations as low as 700 ppb have been demonstrated previously (Mutteres et al. 1993). These observations are consistent with studies demonstrating that aldehyde-containing VOCs cause damage in plants (Almeras et al. 2003; Splivallo et al. 2007). Interestingly, in my study seed germination and seedling formation were not inhibited by formaldehyde.

Localized cell death in plant tissue is indicative of oxidative bursts caused by over abundance of ROS as a response to stress (Heller & Tudzynski 2011; Bhattacharjee 2012). Physiological and biochemical changes such as reduction of leaf size, leaf wilting, changes in relative water content, electrolyte leakage, and production of ROS all aid in preserving cell viability in plants (Bartels & Sunkar 2005; Anjum et al. 2011). Drought, salinity, metal and temperature stresses imposed on vegetative stages are known to cause similar effects (Jaleel et al. 2007; Mafakheri et al. 2010). Indicators of plant stress in my study included severe discoloration and curling of leaves, and localized cell death on leaf
tissue. With the exception of ethanol, when compared to controls, all the VOCs I studied gave rise to significantly lower levels of chlorophyll in treated plants. It has been suggested that plants reduce chlorophyll in order to decrease the overall energy absorption in the photosynthetic apparatus that drives the production of reactive oxygen species (Ommen et al. 1999; Splivallo et al. 2007; Mafakheri et al. 2010).

Volatile effects on plants have been studied most extensively in the context of host-pathogen interactions. For example, the volatile, lipid-derived molecules acrolein and methyl vinyl ketone are potent stimulators of expression of the pathogenesis-related gene HEL (PR4) in A. thaliana (Almeras et al. 2003). Oxylipins, a class of lipid-derived compounds are often released by plants in response to herbivory or pathogen attack, and oxylipins in the jasmonate family play key roles as plant regulators (Farmer et al. 2003). The C-8 family of VOCs we studied are oxylipins characteristic of fungal metabolism (Tsitsigiannis & Keller 2007) and deserve more study as possible toxigenic signaling agents in plants.

Of all the compounds we examined, the most phytotoxic compound was 1-octen-3-one. The biological activity of this particular ketone is not well documented even though it is widely used as flavoring agent and adjuvant. In A. thaliana, it almost completely inhibited seed germination and was lethal to vegetative phase plants. In less than 72 hours, the plants exhibited permanent wilting accompanied by leaf curling and warping. The edges of the leaves quickly turned brown, then yellow and then completely white (bleached). Certain VOCs are known to act as growth-regulating substances during the seedling development process (Ogura et al. 2000; Cape 2003). My studies suggest that 1-octen-3-one might be a useful inhibitor compound to use in biochemical
dissections of the germination process. Furthermore, the phytotoxicity we observed here suggests that 1-octen-3-one and a variety of commercial air freshener products should receive more study in future toxicological testing of flavor and fragrance products.

In conclusion, I have demonstrated the sensitivity of germinating seeds and two week old *A. thaliana* plants to the presence of a variety of anthropogenic and biogenic VOCs at concentrations lower than found in some experiments (Singer et al. 2006). These studies broaden the range of known biological effects of C-8 hydrocarbons and several other low molecular weight volatiles on plant growth and stimulate numerous questions about the mechanism by which low concentrations of VOCs impact plant health. They also add to our store of knowledge about the way in which fungi and plants interact through transkingdom signaling systems that sometimes involve stress responses mediated through volatile compounds (Ortiz-Casro et al. 2009). As a model system with many genetic, genomic and proteomic resources, *A. thaliana* is well suited for more detailed studies at the molecular level.
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concentrations and subjective symptoms associated with sick building syndrome in newly built houses in Japan. Int Arch Occup Environ Health 83: 225-235


Table 1. Volatile compounds used in this study. a: Names, structures, and concentrations of individual compounds. b: Description, odor and available safety information for fragrance products

Footnotes: a) CAS=Chemical Abstracts Service Registry Number b) MSDS=Materials Safety Data Sheet

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<td>stick to aid fragrance</td>
<td></td>
<td>• Proprietary Fragrance mixtures</td>
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<tr>
<td></td>
<td></td>
<td>dispersal</td>
<td></td>
<td>• Proprietary Fragrance mixtures</td>
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<tr>
<td></td>
<td>Scented Body Mist/Spray</td>
<td>Aerosol spray canister; apply</td>
<td>Lemon, citrus</td>
<td>Not available</td>
</tr>
<tr>
<td></td>
<td></td>
<td>directly onto body or</td>
<td></td>
<td>• Proprietary Fragrance mixtures</td>
</tr>
<tr>
<td></td>
<td></td>
<td>clothing</td>
<td></td>
<td>• Proprietary Fragrance mixtures</td>
</tr>
<tr>
<td></td>
<td>Scented Oil</td>
<td>Equipped with micropore</td>
<td>Tropical fruit</td>
<td>Prolonged exposure may cause dizziness, headache, and irritation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>membrane to allow slow</td>
<td>mix</td>
<td>of respiratory tract; irritation of skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>release of scented oils over</td>
<td></td>
<td>• Proprietary Fragrance mixtures</td>
</tr>
<tr>
<td></td>
<td></td>
<td>time</td>
<td></td>
<td>• Benzyl acetate</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Bornyl-3-one (camphor)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• 3,7-dimethyl-2,6-octadien-1 (citral)</td>
</tr>
</tbody>
</table>
Fig 1. VOC exposure setup. A: Germination and seeding formation. B: Vegetative plant exposure
Fig 2. *Arabidopsis thaliana* seeds. A. No germination, B. Germination, C. Progression to seedling.
Fig 3. *Arabidopsis thaliana* exposed to VOCs at 1 ppm for 72 hours. Average percentage of seed germination and seedling development. Treatment was replicated three times and the experiment was repeated four times. N = 30. Error bars represent the standard deviation of the mean, where ANOVA $P = 0.0001$. 
Fig 4. Fourteen-day-old *Arabidopsis thaliana* exposed to VOCs at 1 ppm for 72 hours. Visible indicators of plant stress to VOCs include smaller plant and leaf size, discoloration of leaves, small necrotic lesions, and complete death of plant. A: Plants exposed to common anthropogenic VOCs compared to control. B: Plants exposed to common biogenic VOCs compared to control. C: Exposure to 1-octen-3-ol causes necrotic lesions in plant leaves (red arrow).
Fig 5. Total chlorophyll concentration of *Arabidopsis thaliana* exposed to VOCs at 1 ppm for 72 hours. A: Comparison of all single VOC compounds. B: Comparison of anthropogenic fragrance VOCs in mixtures. Treatment was replicated three times and the experiment was repeated three times (N = 12). Error bars represent the standard deviation of the mean (ANOVA, *P* = 0.0001)
CONCLUSIONS

Plant and bacterial volatile organic compounds (VOCs) are known to influence plant growth but less is known about the physiological effects of fungal VOCs. I have used Arabidopsis thaliana as a model to test the effects of VOCs from the soil fungus Trichoderma viride as well as other fungi. Compared to controls, plants grown in the presence of T. viride volatiles were taller, bigger, flowered earlier, and had more lateral roots.

GC-MS was conducted using two different resins to determine the profile of T. viride at the time of exposure. A total of 56 different compounds were adsorbed by Tenax and Super-Q resins. A. thaliana seeds and vegetative plants were exposed to 23 of the 56 compounds at one part per million (ppm) volume to volume. No compound tested induced the growth promoting effects observed in the T. viride exposure tests.

The different outcome is likely due to three possibilities. The first possibility is that the volatiles were used at an incorrect concentration. In insect volatile signaling, a difference of one log volatile concentration can determine the outcome of an exposure: attraction, no reaction, and repulsion. A similar specificity is observed in tests that exposed A. thaliana seeds to 1, 2, and 3 ppm of enantiomers of 1-octen-3-ol. Each increase of 1ppm of 1-octen-3-ol resulted in a larger percentage of seeds that were unable to complete seedling formation. A second possibility is that the 23 volatiles tested were not responsible for the effects observed in the T. viride tests. A different volatile maybe the causitive agent. The third possibility is that there is no single VOC induces growth promotion, instead, a mixture of compounds is necessary. It is possible that there are a
number of effects are induced in the plant simultaneously resulting in increased biomass production and chlorophyll concentration.

The sensitivity of plants to VOCs and their inability to move also make them good candidates for use as biosentinels; organisms that are able to detect and relay information about something of interest. There has been a study to use roadside weeds as a sentinel that can react to the presence of explosives for the detection of roadside bombs. The group Botanic Gardens Conservation International has plans to use plants as sentinels for the detection of agricultural pests and pathogens. Plants can also be used to identify areas that have poor air quality either due to fungal infestation or unhealthy concentrations of anthropogenic pollutants such as fragrance products. Exposure to the 1ppm of the volatilized form of popular and common fragrance products have had detrimental effects on *A. thaliana* such as chlorosis and inhibition of seedling formation.

This study along with others have shown that *A. thaliana* and other plants to react to fungal VOCs and other volatile compounds. Future experiments include exposure studies conducted on tomato plants, with different fungal VOCs, and chemical VOCs. Data on change in genetic expression in *A. thaliana* exposed to *T. viride* has been collected through the use of a microarray and will be annotated. This will show what pathways are affected by VOC exposure and may provide a clue to determining the VOC(s) responsible for growth promotion. Additional expression studies are being conducted to verify the microarray as well as detail the effects of other fungal VOCs and chemical VOC standards. I have only begun to unravel the effects of fungal VOCs on plants. This interaction is still a poorly understood and should be further investigated.