ANALYSIS OF VOLATILE ORGANIC COMPOUNDS EMITTED BY FILAMENTOUS FUNGI AND VOLATILE-MEDIATED PLANT GROWTH

by

SAMANTHA YUN JENG LEE

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Joan W. Bennett, Ph.D.

And approved by

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ABSTRACT OF THE DISSERTATION

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Volatile organic compounds (VOCs) are a small portion of the total metabolites produced by organisms; however, their unique properties enable them to mediate important biological functions, especially in aerial and terrestrial environments. In agriculture, the potential uses of VOCs include volatile-mediated inhibition of pathogen growth and increased plant systemic resistance. Filamentous fungi in the genus *Trichoderma* are robust biological control agents as they utilize several modes of action including resistance, antibiosis, competition and myco-parasitism. Earlier work by our laboratory demonstrated the ability of *Trichoderma*-derived VOCs to stimulate *Arabidopsis* growth.

In this dissertation, *Trichoderma* emission profiles, concentrations, quantities, and VOC-mediated effects on plants were measured in order to develop a mechanistic understanding of the volatile-mediated *Trichoderma*-to-plant interactions. *Trichoderma* volatile-mediated plant growth promotion was dependent on the age of the fungal culture,

developmental stage of the plant, duration of the exposure, and was isolate-specific. Screening of 20 *Trichoderma* isolates for VOC-induced growth of *Arabidopsis* identified 9 growth promoting isolates increasing plant biomass (up to 41.6%) and chlorophyll content (>89.3%). In addition, similar responses to VOC mixtures were obtained in tomatoes, i.e. a significant increase in plant biomass (>99%), larger plant size, and significant development of lateral roots, suggesting that plant growth promotion may occur through a similar mode of action in different types of plants. GC-MS analysis of VOCs from *Trichoderma* isolates led to identification of more than 147 unique compounds and several unknown sesquiterpenes, diterpenes, and tetraterpenes.

After VOCs were identified from GC-MS data, 26 compounds were selected and tested individually on *Arabidopsis* in order to determine if individual compounds could mimic the effects of *Trichoderma* volatile mixtures on plant growth. Of the compounds tested, exposure to 1-decene yielded increased seed germination, plant fresh shoot weight (>38.9%), and chlorophyll (>67.8%). RNA sequencing analysis of *Arabidopsis* shoots treated with 1-decene for 72 hours identified 123 differentially expressed genes involved in volatile responses. Up-regulated genes were related to growth, responses to hormone and cell wall modifications inducible by auxin. The RNA-seq data provides a list of candidate genes to screen in future research on the biological activities of fungal VOCs.

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CHAPTER 1.

INTRODUCTION

The author is currently preparing to submit a book chapter.

1.1 Overview of biogenic volatile organic compounds (VOCs)

The study of biogenic volatile organic compounds (VOCs) and their physiological effects has intensified in recent decades. Although volatiles are a small portion of the total metabolites produced by organisms, their unique properties enable them to mediate important biological functions, especially in aerial and terrestrial environments. There also exists a large body of literature investigating volatiles including their roles as food and flavoring agents, semio- or info-chemicals for insects and microbes, and as indicators of microbial contamination. In agriculture, the potential use of microbial VOCs include biostimulation of crops, control of contamination in food products, and control of pathogens in plants through volatile-mediated inhibition of pathogen growth and/or increased plant systemic resistance. Our current knowledge of the VOCs emitted by microorganisms will continue to grow as we obtain new volatile profiles, accurately assess the impact of environmental conditions on volatile production, and identify novel compounds. Moreover, elucidating the biological activities and ecological roles of VOCs will become increasingly important as we seek to develop more sustainable agriculture practices. The scope of this chapter is to summarize what is known about the VOCs produced by microbes and the complex volatile-mediated interactions between plants, fungi, and bacteria in the soil environment, with the intent to offer insight into the complexity of the roles and functions of these versatile compounds.

The production and emission of VOCs have been an important area of research in studies of atmospheric and terrestrial ecosystems. VOCs have low molecular mass, high vapor pressure (>0.01 k Pa), low boiling point and low polarity. These characteristics allow compounds to vaporize and diffuse through air and air spaces in soil (Insam and

Seewald, 2010; Penuelas et al., 2014). Plants and microorganisms contribute to the total VOCs detected in soil. Several thousand VOCs emitted by plants have been identified. In comparison, only several hundred compounds have been identified from microorganisms; however, this number continues to grow as more microbes are assessed for their volatile production. The microbial volatile profile is dependent on multiple parameters such as geography, time, and environmental conditions including nutrient content, microbial community composition, temperature, humidity, and pH (McNeal and Herbert, 2009; Insam and Seewald, 2010). These factors lead to drastically different emission profiles, concentrations, and quantities observed in nature. It is becoming increasingly apparent that there are species- and isolate- specific differences in the production of volatiles. Several taxonomic studies have identified and characterized microorganisms by means of a combination of morphological, molecular, and volatile characteristics. In turn, this has led to using microbial volatile detection as a diagnostic tool to measure microbial contamination of food products, food spoilage, infection, and environmental contaminants such as growth in buildings with moisture problems (Wilkins et al., 2000; Wheatley, 2002; Mayr et al., 2003; Karlshoj et al., 2007; Korpi et al., 2009).

Much of the early work has focused on plant VOCs, structures, and functions especially in relation to plant catabolism and degradation, and their effects on the atmosphere and air quality (Oikawa and Lerdau, 2013). Plant VOCs are important in mediating plant-to-plant as well as plant-to-insect communications. Many plants release volatiles in response to wounding and these compounds act as chemical signals to the neighboring plants, inducing defensive responses. Plant emitted VOCs can lead to the

reduction and avoidance of foliar damage (Farag et al., 2005; Heil et al., 2007; Matsui et al., 2012). Examples of well studied plant volatile signaling compounds used in plant response and development include ethylene, methyl jasmonate and β-ocimene (War, 2011; Santino et al., 2013; Groen and Whiteman, 2014; Menzel et al., 2014). There is an ecological and physiological advantage in recognizing volatile signaling compounds, where the catabolism of the volatile enables plants to prime themselves for pathogen attack while avoiding the cost of constitutive defense (Oikawa and Lerdau, 2013).

The production of VOCs by plants and microorganisms, their measurements, and biological effects have been discussed in several reviews (Stotzky and Schenck, 1976; Linton and Wright, 1993; Cape, 2003; Tholl et al., 2006; Korpi et al., 2009; Insam and Seewald, 2010; Morath et al., 2012; Bitas et al., 2013; Dudareva et al., 2013; Oikawa and Lerdau, 2013; Penuelas et al., 2014; Hung et al., 2015).

1.2 Environmental factors that affect microbial volatile production and emission in soil

Microorganisms living in the soil are affected by the soil community composition, nutrient and oxygen availability, and the physiological state of the microorganisms. Oxygen availability is influenced by the physical properties of soil, substrate quality and texture, and moisture. Organic matter contributes to the formation of soil pore structures, affecting the diffusion and adsorption of VOCs in soil (van Roon et al., 2005a; 2005b; Hamamoto et al., 2012). Adsorption of VOCs is dependent on soil texture and in general, polar compounds are more strongly adsorbed than aliphatic and aromatic compounds (Ruiz et al., 1998). Alkaline soil tends to increase adsorption of VOCs compared to acidic soil (Serrano and Gallego, 2006). Nutrient conditions such as type of nitrogen

source available, along with the presence of other microbes, lead to drastically different volatile production by resident microbes. The pH of the soil impacts the nutrient availability and directly affects the physiological state of the organisms, ultimately changing metabolite production. Moisture, pH, and temperature affect VOC retention in soil and the soil itself can act as a sink for VOCs (Asensio et al., 2007; Ramirez et al., 2009). Lastly, the chemical property of the volatile, its vapor pressure and water solubility affect the retention properties of VOCs in soil.

Microorganisms and plant root mass contributes to the large amount of organic matter in soil. A wide range of intermediate and end products of fermentation and respiration are generated as microbes decompose organic matter such as leaf litter or root exudates. Microorganisms can consume VOCs as a carbon source and the degradation of VOCs in turn impacts the volatile composition of soil (Owen et al., 2007; Ramirez et al., 2009). For example, VOCs such as formic acids and acetic acids are constantly being removed and degraded by microorganisms living in the anaerobic and aerobic microhabitats within the soil environment (Del Giudice et al., 2008). VOCs emitted by roots are also modified by bacterial and fungal colonizers as they break them down into a carbon source. In general, as VOCs solubilize in the liquid phase or are adsorbed to the soil surfaces, microorganisms are able to degrade the volatiles, converting them into metabolic products (Malhautier et al., 2005; Owen et al., 2007).

1.3 VOC production from soil microorganisms

VOC production by soil microorganisms is often studied under controlled conditions in order to minimize and control the complex differences in soil, microbial community, and soil properties found in natural environments. There are only a limited

number of studies characterizing total VOC productions in a soil habitat. When root-free soil and litter samples were compared for volatile emissions, only 13 compounds out of 100 total VOCs captured were positively identified in the soil sample while 64 compounds were identified in the litter sample (Leff and Fierer, 2008). Although this study did not identify the specific microorganism responsible for the volatile production, there was a strong correlation between microbial biomass and volatile production rates. In general, soil emissions are dominated by the presence of terpenes, terpenoids, and oxygenated VOCs such as methanol, acetaldehyde, and acetone (Schade and Goldstein, 2001; Schade and Custer, 2004; Asensio et al., 2007; Greenberg et al., 2012). Some volatiles such as furfural and furan compounds are also found in high concentration. Furfural is emitted by soil fungi and typically found in decomposing leaf litter (Stotzky and Schenck, 1976; Isidorov and Jdanova, 2002; Leff and Frierer, 2008). Several intermediate products of microbial metabolism such as propanoic, acetic, and butanoic acids are also present in soil.

Soil VOCs are potential indicators of microbial community structure and community shifts (McNeal and Herbert, 2009). Volatile analyses of different soil habitats i.e. saline coastal upland, seasonal wetland, and grassland area, have been done. Environmental factors such as the abundance of microorganisms, substrate and water availability, and soil texture differ among the soil samples tested. Of the 72 VOC metabolites identified by McNeal and Herbert (2009), there were significant differences in the estimated number and types of compounds produced between soil types. Microbial VOC production and CO_2 evolution increased over time and presented a strong correlation between VOC patterns and community levels and structures.

It has been suggested that the specific pattern of VOCs emitted by different microbes can be used for taxonomic purposes. Recently, a database of bacterial and fungal volatiles has been compiled and is available online at http://bioinformatics.charite.de/mvoc (Lemfack et al., 2014). In general, the dominant classes of compounds emitted by bacteria are alcohols, alkanes, alkenes, ketones, esters, pyrazines, lactones, and sulfides. For example, gram-positive bacteria such as *Lactococcus lactis* produce butyric acid, dimethyl sulfide, isoprene, and butanone (Mayrhofer et al., 2006). Gram-negative bacteria such as *Pseudomonas*, *Serratia*, and *Enterobacter* produce species-specific dimethyl disulfides, dimethyl trisulfides, and isoprenes (Schöller et al., 1997).

Fungi typically produce alcohols (e.g., isomers of butanol, pentanol, octanol), hydrocarbons, ketones, terpenes, alkanes, and alkenes (Effmert et al., 2012). Moreover, certain compounds such as 3-methyl-1-butanol and 1-octen-3-ol are widespread among fungi studied so far. However, some compounds appear to be uniquely produced by certain species and/or isolates and these species are distinguishable based on volatile production alone. To take advantage of these distinctive traits, VOCs have been exploited in chemosystematics to supplement morphological and molecular identification techniques. For example, when the VOCs of 47 different taxa of *Penicillium* were studied, more than half of the volatile metabolites were only detected from one taxon (Larsen and Frisvad, 1995a; 1995b). Several *Aspergillus* species commonly found in water-damaged building were analyzed for their volatile emissions and also showed species-specific VOC production (Polizzi et al., 2012; Lee et al., 2015b). *Aspergillus* spp. grown on malt extract agar differed in sesquiterpene production. In particular, *A. versicolor*, *A. ustus*, and *Eurotium amstelodami* differed in VOC pattern whereas

Chaetomium spp. and *Epicoccum* spp. were differentiated by their volatile production from a group of 76 fungal strains belonging to different genera (Polizzi et al., 2012). Many *Trichoderma* emit similar C6 - C8 compounds; however, different *Trichoderma* spp. and isolates tend to differ drastically in the production of terpenes and terpenoids especially sesquiterpenes (Fiedler et al., 2001; Lloyd et al., 2005; Siddiquee et al., 2012; Lee et al., 2015a).

1.4 Detection of VOCs

Several techniques are available for volatile detection and quantification. Advanced high-throughput analysis and statistical tools are continuously being developed and modified to enable researchers to obtain high quality data and improved data normalization methods. However, there are also methodological challenges associated with the analysis of soil and microbial samples. For example, one of the major limitations of analytical systems is condensation and/or adsorption of heavy semi-volatile and non-volatile compounds. A few important techniques used to detect VOCs are briefly summarized below.

One type of analytical method involves a direct connection between the analytical apparatus and the setup used to generate the gas samples to be measured (Comandini et al., 2012). Since it is employed in the field, the fast analysis of the sample provides the advantage of detecting short-lived species such as radicals and larger compounds that readily oxidize, condense, or are adsorb by various surfaces. However, it cannot be used when dealing with large volumes or high pressured samples.

Proton transfer reaction – mass spectrometry (PTR-MS) is a technique for real-time monitoring of VOCs without sample preparation. The PTR-MS technique is highly

sensitive and can detect low concentrations of VOCs (parts per trillion volume [pptv]) in air and gas samples. This method has been used to detect microbial VOCs in food, degradation of organic waste, and soil samples (Hansel et al., 1995; Mayr et al., 2003; Mayrhofer et al., 2006; Asensio et al., 2007; Seewald et al., 2010). PTR-MS is designed only for trace gas detection and is not suitable for direct liquid sample analysis.

Membrane inlet mass spectrometry (MIMS) separates compounds from air and water samples using a thin silicone membrane. In the MIMS sampling probe, the silicone membrane is placed between the sample and the ion source of a mass spectrometer. MIMS technique is quick and easy to use, and allows on-site analysis of VOCs (Wong et al., 1995; Ketola et al., 2011). MIMS is suitable for measuring VOCs spatially and temporally from soil and water samples, slurry samples, and other types of solid samples. MIMS has been utilized to study soil and microorganism turnover processes (Lloyd et al., 2002; Sheppard and Lloyd, 2002; Schluter and Gentz, 2008).

Alternatively, samples can be collected prior to analysis over a relatively long time, at a different location from the analytic instrumentation. However, such techniques are limited to measuring stable compounds that can be easily collected and stored. To detect large and heavy compounds, additional procedures need to be implemented prior to analysis such as using a resin trap or rotary evaporator (Winberry Jr. and Jungclaus, 1999; Comandini et al., 2012).

The identification of VOCs by head space or thermal desorption gas chromatography (GC) uses different columns in combination with appropriate detection methods: mass spectrometry (MS), flame ionization detector (FID), flame photometric detector (FPD), infrared analyzer (IR), or photoionization detector (PID) (Moeder, 2014;

Hubschmann, 2015). Each type of GC column is selective for specific chemical groups, so no single one is capable of total VOC estimation. Analysis by GC-MS requires pre-concentration of VOCs in adsorption traps comprised of hydrocarbon or other adsorbents packed in stainless steel or glass tubes. The air sample is moved through the adsorbent tube, and the compounds are trapped inside. Once the compounds are collected, the trap is thermally desorbed at high temperature. Individual compounds are then identified using a database (library) of mass spectra or by comparing retention times and spectra with known standard compounds. Currently, GC-MS is the dominant method used to characterize volatile profiles from soils, housing materials, as well as microbial and plant samples (Serrano and Gallego, 2006; Leff and Fierer, 2008; Betancourt et al., 2013).

Solid-phase microextraction (SPME) is a solvent-free adsorption and desorption technique where desorption occurs in the GC injector. It consists of fused-silica fibers coated with different polymers to isolate and concentrate chemicals based on equilibrium. It is relatively quick, easy to use, and practical; the extraction, concentration and introduction are in a single step (Basheer et al., 2010). The primary limitation is the reduced adsorption capacity of the fiber due to the small volume of polymer coating on the fiber. For example, heavier materials can be preferentially adsorbed into fibers (displacement rate) and extract preservation is not possible. Combining SPME with other techniques, such as SPME GC-MS, has been successful in profiling living fungal, plant, and soil samples (Jassbi et al., 2010; Stoppacher et al., 2010; Tait et al., 2014).

It is important to note that most of the compounds found in volatile libraries available today are comprised of volatile chemical identified from animals and plants. Potentially, there are many unknown VOCs emitted by microorganisms yet to be

identified. In order to identify and determine structures of new compounds, analytical methods such as nuclear magnetic resonance (NMR) spectroscopy are used For example, NMR spectroscopy was used to identify harziandione, a diterpene, from *Trichoderma harzianum* (Miao et al., 2012) and sodorifen from the bacterial species, *Serratia plymuthica* (Kai et al., 2010). The combination of these analytical methods in addition to techniques to identify and characterize new compounds will continue to provide a comprehensive profile of microbial VOCs.

1.5 Volatile-mediated interactions between plants, bacteria, and fungi

Chemical ecologists have discovered that many plant, bacterial, and fungal VOCs have potent physiological effects where they function in signaling, communication, antagonism, and inter- and intra-specific associations. VOC-mediated effects, their biological and ecological significance, and their role in the development of soil ecosystem have received increased attention in recent years (Bitas et al., 2013; Davis et al., 2013; Penuelas et al., 2014; Hung et al., 2015).

VOCs differ significantly in structure and function where a single compound can affect numerous aspects of an organism's growth and development. For example, dimethyl disulfide, which is produced by plants and microorganisms, has multiple functions as an insect attractant, elicitor for plant systemic resistance, and suppressor of pathogenic fungi (Kai et al., 2007; Crespo et al., 2012; Huang et al., 2012). Mixtures of microbial VOCs play a role in the formation and regulation of symbiotic associations and in the distribution of saprophytic, mycorrhizal, and pathogenic organisms in the soil environment (Bonfante and Anca, 2009; Rigamonte et al., 2010; Muller et al., 2013).

1.6 VOCs from plant roots

Plants release volatile mixtures from leaves and roots during herbivore and pathogen damage. For example, methanol production in plants has been linked to herbivore and pathogen damage and known to modulate plant defense responses (Hann et al., 2014). Methanol is produced during the cell wall modification completed by the activity of pectin methylesterases; it is primarily associated with leaf expansion, cell elongation and root elongation (Galbally and Kirstine, 2002; Hueve et al., 2007; Palin and Geitmann, 2012). Foliar application of methanol reduces stress and affects growth, as well as fruit productivity and quality, in several plant species. It has been suggested that methanol application may increase plant growth as a carbon source and increase photosynthesis efficiency (Nonomura and Benson, 1992; Ramadan and Omran, 2005; Ramirez et al., 2006; Mahalleh Yoosefi et al., 2011; Paknejad et al., 2012; Bagheri et al., 2014). In soil, many methylotrophic species use the methanol produced by plant roots as a carbon source (Kolb, 2009). A few filamentous fungi (*Aspergillus niger* and *Trichoderma lignorium*) and several yeast isolates (*Hansenula*, *Candida*, *Pichia*, *Torulopsis*, *Kloeckera*, and *Saccharomyces*) also have the ability to obtain energy from the oxidation of reduced one-carbon compounds like methanol. There is a trade-off between plants and microbes, these methylotrophic bacteria and fungi promote plant growth by aiding in nutrient uptake and the production of plant hormones (Hanson, 1992; Iguchi et al., 2015).

When plants are being challenged by biotic factors such as insect pests, they emit a diverse range of compounds, including the release of short-chain C6 and C9 volatiles, aldehydes, and terpenes. In turn, the VOCs released into the soil by damaged plants have

direct bactericidal and fungicidal activities. Terpenes such as β-caryophyllene and β-phellandrene function as inter- and intra-plant signals and inhibit the spread of pathogens by attracting beneficial organisms, thus indirectly creating defense response (Prost et al., 2005; Frost et al., 2007). Herbivory-induced plant root VOCs attract predatory insects, mites, parasitoids, and nematodes that feed on the pests. For example, dimethyl disulfide is produced by *Brassica nigra* roots during pathogen infection attracts soil-dwelling beetles that are natural predators of root fly larvae (Ferry et al., 2007; Crespo et al., 2012).

1.7 VOCs from bacteria

Soil microorganisms are ubiquitous and have been studied intensely for their effects on plant growth and development, recycling of biomass in the environment, and interactions between organisms. Many soil bacteria are found in biofilms on plant roots, leaf litter, and soil particles (Burmølle et al., 2007). In the rhizosphere, plants release root exudates that affect microbial composition by providing a nutrient rich habitat that is colonized by mycorrhizal fungi and associated mycorrhization helper bacteria (Bonfante and Anca, 2009; Rigamonte et al., 2010). Although VOCs can serve as an energy source, these compounds are especially important in competitive and symbiotic conditions such as mycorrhiza formation. Soil diseases are suppressed when plants and microorganisms release antibacterial and antifungal compounds thus preventing pathogen attacks. The release of bacterial VOCs affects antibiosis and signaling resulting in beneficial (stimulatory) or detrimental effects.

Several bacterial isolates have been studied for their volatile-mediated fungistatic activities. In the laboratory, these bacterial VOCs reduce the growth of fungal cultures

and inhibit spore germination. The degree of inhibition is largely dependent on environmental constraints, the age of the fungal culture, and the species tested. Chuankun et al. (2004) examined the suppressive effects of the VOCs emitted from fungistatic soil and measured the growth of several fungi. Following sterilization of the soil, the inhibitory effects disappeared suggesting the importance of microbial activities in the fungistatic process. Of the VOCs identified, trimethylamine, benzaldehyde, and N,N-dimethyloctylamine all exhibited very strong antifungal activity at low concentration.

Over the years, a variety of *in vitro,* small scale volatile exposure methods have been developed to study volatile-mediated interactions between bacteria, fungi and plants (Fig. 1). Bacteria studied for VOC emissions and volatile-mediated effects includes but is not limited to, *Bacillus subtilis, Pseudomonas fluorescens, Pseudomonas trivialis*, *Burkholderia cepacia*, *Staphylococcus epidermidis*, *Stenotrophomonas maltophilia*, *Stenotrophomonas rhizophila*, *Serratia odorifera*, and *Serratia plymuthica*. The mixtures of bacterial VOCs emitted by growing cultures inhibit the mycelial growth of many pathogenic fungi including *Aspergillus niger*, *Fusarium culmorum*, *Fusarium solani*, *Microdochium bolleyi*, *Paecilomyces carneus*, *Penicillium waksmanii*, *Phoma betae*, *Phoma eupyrena*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Trichoderma strictipile*, and *Verticillium dahlia* (Kai et al., 2007; Vespermann et al., 2007).

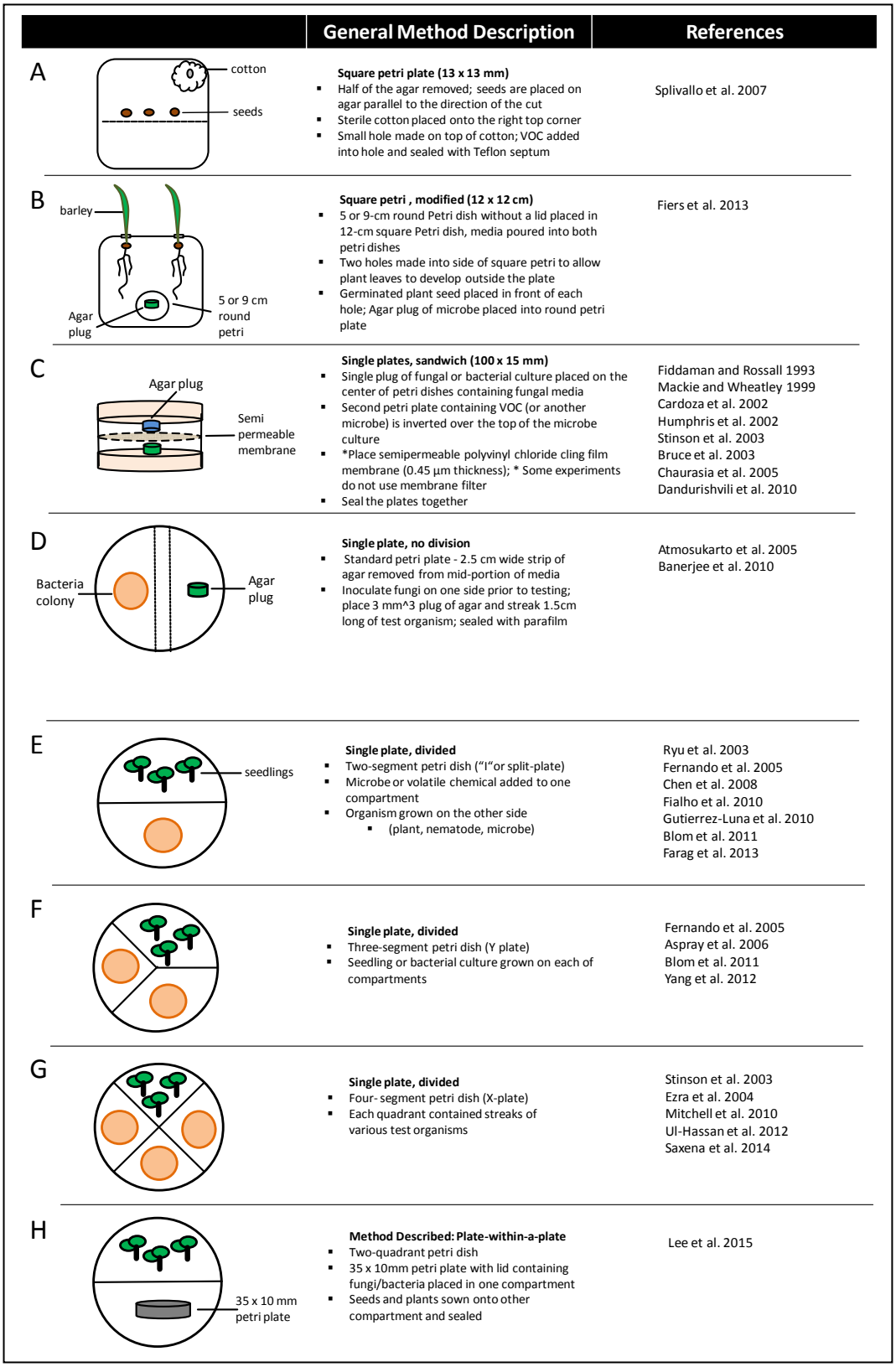

Figure 1. Overview of volatile exposure methods to study volatile-mediated interactions.

In some cases, specific volatile compounds from bacterial mixtures that cause the fungal growth inhibition have been identified. These compounds are diverse and include C7 (benzothiazole), C8 (1-octen-3-ol and 2-ethylhexanol), C9 (nonanal), C10 (decanal), C11 (1-undecene), terpene (citronellol), nitrogen containing (trimethylamine), and sulfur containing (dimethyl disulfide) (Chitarra et al., 2004; Fernando et al., 2005; Kai et al., 2009). The aromatic heterocyclic compound (benzothiazol), benzene derivative (2-phenylethanol), and phellandrene derivative ((+)-epi-bicyclesesquiphellandrene) also reduce mycelial growth and spore germination of pathogenic fungi (Wan et al., 2008; Zhao et al., 2011).

In contrast to the increasing knowledge of bacterial volatiles' suppression of fungal growth, not as much has been documented about fungal growth promotion or the impact of these VOCs on the bacteria themselves. The effects of bacterial volatiles on fungi include stimulation of fruiting body formation and spore germination of *Sclerotium*, *Rhizoctonia*, and *Agaricus* (Kai et al., 2009). A few studies have examined the effects of bacteria-on-bacteria volatile-mediated interactions of *Clostridium perfringens*, *Veillonella* spp., *Bacteroides fragilis*, and *Burkholderia cepacia* (Hinton and Hume, 1995; Wrigley, 2004).

Bacteria interact with plants through VOCs to either promote or inhibit plant growth. One early study demonstrated that several species of plant growth-promoting rhizobacteria (PGPR) improve plant growth by emitting growth promoting VOCs (Ryu et al., 2003). Blends of VOCs from *Bacillus subtilis* GB03 and *B. amyloliquefaciens* produced the greatest effects in plant growth; moreover, theses bacteria were the only ones that produced 2,3-butanediol and acetoin. Direct application of 2,3-butanediol

enhanced plant growth similarly to VOC mixtures. Since then, additional rhizobacterial species and their volatiles were evaluated for their effects on fungal and plant growth (Vespermann et al., 2007; Kai et al., 2010). Exposure to *B. cepacia* and *S. epidermidis* increased plant size while exposure to *B. subtilis* B2g VOCs had no significant effect on plant development. However, *Arabidopsis* exposed to *P. fluorescens*, *P. trivialis*, *S. plymuthica*, *S. odorifera*, *S. rhizophila*, and *S. maltophilia* exhibited significant growth inhibition and death.

To determine how the VOCs from PGPR trigger growth in plants, the RNA transcripts of *Arabidopsis* exposed to *B. subtilus* were examined using a microarray analysis (Zhang et al., 2007). More than 600 differentially expressed genes were identified, including many genes involved in auxin homeostasis, underling the importance of auxin in regulating cell expansion in plants. A screen of rhizosphere bacteria and bacterial volatile-mediated effects on *Arabidopsis* ranged from plant death to a six fold increase in plant biomass and were dependent on several factors including cultivation medium and inoculum quantity. More than 130 VOCs were identified and indole, 1-hexanol, and pentadecane were further tested on plant growth. Although none of these compounds triggered a defense response in plants, the compounds appeared to act as effectors to inhibit the plant defense response (Blom et al., 2011).

1.8 VOCs from fungi

The functionality of fungal volatiles is gaining attention in agricultural, environmental, and ecological studies. Fungal VOCs have been exploited to detect contamination in food processing and indoor environments, and in assessing health risks (Korpi et al., 2009; Heddergott et al., 2014). They also have been used to suppress

pathogenic bacteria and fungi. In a process called "mycofumigation", the VOCs from endophytic fungi in genus *Muscodor* killed several pathogenic fungi and bacteria (Strobel et al., 2001; Mitchell et al., 2010; Kudalkar et al., 2012; Alpha et al., 2015). Fewer studies have explored volatile-mediated interactions between plants and fungi. VOC emission profiles are dynamic and can change during fungus-to-fungus interactions. As a given species recognizes and reacts to the presence of another fungus, the production of VOCs may alter and impact growth. For example, when the mycelia of wood decay basidiomycetes fungi, *Hypholoma fasciculare* and *Resinicium bicolor* interacted physically, a new set of sesquiterpenes such as α-muurolene and γ-amorphene were produced (Hynes et al., 2007). Hence, the fungal volatile profile can provide insight into the microbial activities and community structures especially in root-associated fungi. The volatile profiles of ectomycorrhizal, pathogenic, and saprophytic fungal species differ in their volatile profiles and pattern of sesquiterpene productions. Using emission patterns, different ecological groups can be predicted with 90-99% probability (Muller et al., 2013).

The antibiotic activities of *Trichoderma* VOCs have been documented early on (Dennis and Webster, 1971). Several species of *Trichoderma* (*T. harzianum*, *T. viride*, *T. lignorum*, *T. hamatum*, and *T. reesei*) are able to inhibit the growth of *Fusarium solani*, a phytopathogen (Chakraborty and Chatterjee, 2008). Several volatile and non-volatile compounds from *Trichoderma* species significantly reduced the growth of the pathogen. Non-volatile compounds induced complete inhibition while volatile compounds inhibited the growth of *F. solani* up to 78%. Similarly, *T. pseudokoningii* VOCs suppressed spore

germination and mycorrhiza establishment of the mycorrhizal species *Gigaspora rosea* in soybean (Martinez et al., 2004).

Antimicrobial volatiles produced by fungal endophytes have potential as agricultural biocontrol agents. The VOCs from *Muscodor albus* kill several pathogenic fungi and bacteria (Strobel et al., 2001). Attempts to reproduce the effect with individual components of the VOC blend such as 1-butanol and 3-methyl-acetate inhibited pathogen growth but did not result in complete death as observed in VOC mixture conditions, demonstrating the additive or synergistic mechanism of *M. albus* VOCs. Subsequently, additional species of *Muscodor* were evaluated for their volatile production; *M. crispans* and *M. sutura* produced antibacterial and antifungal VOCs (natural and artificial mixtures) that successfully inhibited the growth of numerous plant and human pathogens (Mitchell et al., 2010; Kudalkar et al., 2012). *Oxyporus latemarginatus* inhibits mycelial growth of pathogenic fungi by producing 5-pentyl-2-furaldehyde (Lee et al., 2009). *Phomopsis* spp. produce sabinene and several other VOCs that possess antifungal properties. Artificial mixtures mimicked similar antibiotic effects against the pathogens *Pythium*, *Phytophthora*, *Sclerotinia*, *Rhizoctonia*, *Fusarium*, *Botrytis*, *Verticillium*, and *Colletotrichum* (Singh et al., 2011).

Another major group of fungi that produce VOCs that can inhibit phytopathogenic species are saprobiontic fungi isolated from the forest and dead wood samples. For example, *Trichoderma viride*, *Schizophyllum commune*, and *Trametes versicolor* all emit VOCs that have up to 20% inhibition of pathogens, *Botrytis cinerea* and *Fusarium oxysporum*. When the mycelial biomass of the saprobionitc fungi was

increased, the negative effects increased and caused 63% inhibition of *Fusarium* and 86% inhibition of *Botrytis* (Schalchli et al., 2011).

As stated previously, VOC profiles can differ between microbial isolates. Therefore, it is not surprising that one isolate can negatively impact another. For example, VOCs emitted by *Fusarium oxysporum* MSA 35 (wild-type strain) suppress the growth of the pathogenic strain of *F. oxysporum* and repress the expression of virulence genes (Minerdi et al., 2009). Volatile analysis revealed that only the wild-type produced sesquiterpenes such as β-caryophyllene, α-humulene, and cyclocaryophyllan-4-ol. Differences in the production of terpenes and terpene derivatives in fungi were also reported in *Aspergillus* (Lee et al., 2015b). Similar strain differences were found when two isolates of *A. versicolor* were studied for their volatile production. The production of sesquiterpenes and diterepenes differed between the two strains and their concentration increased over time. When tested in *A. thaliana*, the volatile mixture from the terpene producing isolate caused significant inhibition in plant growth.

Some fungi produce phytotoxic VOCs. The volatile mixtures from the fruiting bodies of *Tuber* (truffle) species inhibit the growth of *Arabidopsis* (Splivallo et al., 2007). When individual compounds from the mixture of VOCs emitted by truffles were tested on plants, the most phytotoxic compounds were 1-octen-3-ol and trans-2-octanol. It was hypothesized that the truffle VOCs may be the reason for the 'burnt' areas commonly found in nature around truffle-mycorrhized plants.

There is a great deal of interest in identifying the specific compounds within the mixture of VOCs emitted by fungi due to the fact that they induce both positive and negative effects on plant growth. Research to date on individual compounds show that

concentration and duration of volatile exposure play a critical role when measuring beneficial or inhibitory effects. For example, 1-hexanol, a common truffle volatile had a growth promoting effect in plants (Blom et al., 2011). However, at a higher concentration the same compound inhibited plant growth (Splivallo et al., 2007). Several common fungal C8 compounds reduced growth in *Arabidopsis* at a relatively higher concentration (1µl/l [vol/vol]) and 1-octen-3-one, a ketone, killed the plant in 72 hours. (Lee et al., 2014).

Mushroom alcohol, 1-octen-3-ol, is phytotoxic to plants at higher concentrations from 1 to 13 ppm (vol/vol) (Combet et al., 2006; Splivallo et al., 2007; Hung et al., 2014). In contrast, lower concentration of 1-octen-3-ol (0.1 M) enhances resistance to *Botrytis* in *A thaliana* by activating defense genes that are usually turned on by wounding or ethylene and jasmonic acid (JA) signaling (Kishimoto et al., 2007). Produced by *Trichoderma* species, the coconut aroma, 6-n-pentyl-2H-pyran-2-one (6PP) stimulates seedling growth and reduces disease symptoms of *Botrytis* and *Leptosphaeria* (Vinale et al., 2008). Exposure to 6PP also induced an over-expression of pathogenesis-related (PR-1) gene in plants. In another study, *Ampelomyces* sp. and *Cladosporium* sp. produced VOCs that significantly reduced disease severity in *Arabidopsis* plants against the pathogen *Pseudomonas* (Naznin et al., 2013). The two fungi produced m-cresol and methyl benzoate. These compounds elicited induced systemic resistance against the pathogen. Both the salicylic acid (SA) and JA signaling pathways were affected by m-cresol, whereas methyl benzoate was mainly involved in the JA signaling pathway with partial recruitment of SA signals (Naznin et al., 2013).

Biological control strategies involve the usage of beneficial organisms and their products to interfere with pathogens in the environment or otherwise enhance plants growth; several fungal species are widely utilized in agriculture as biological control agents (Butt and Copping, 2001; Gardener and Fravel, 2002; Bailey et al., 2010). It is becoming apparent that in some cases part of the biocontrol effect is mediated by VOCs. Therefore there is a interest in better harnessing the potential of fungal VOCs to enhance growth of plants with agricultural importance and thereby contribute to sustainable farming.

F. oxysporum and its bacterial consortium VOCs stimulate an increase in overall plant biomass and higher chlorophyll content; the sesquiterpene β-caryophyllene is one of the VOCs responsible for the growth-promoting effect (Minerdi et al., 2011). *Cladosporium cladosporioides*, another fungal biocontrol species, enhances growth of tobacco seedlings through its volatile emissions of which α-pinene, β-caryophyllene, tetrahydro-2,2,5,5-tetramethylfuran, dehydroaromadendrene, and (+)-sativene were component parts (Paul and Park, 2013). Further, when *Arabidopsis* plants were grown in a shared atmosphere with *Trichoderma viride* VOCs, the plants were larger with increased lateral roots and earlier flowering (Hung et al., 2013). The genus *Phoma* has a number of plant growth-promoting species that produce C4 - C5 compounds that vary in number and quantity as the culture matures. Exposure to 2-methyl-propanol and 3-methyl-butanol induce growth-promoting effects in *Nicotiana* (tobacco) (Naznin et al., 2013).

In summary, fungal VOCs in mixtures and as individual compounds clearly are able to affect plant growth in positive and negative ways. Nevertheless the underlying

molecular mechanisms in the plants and the ecological relevance of these effects remain largely unknown.

1.9 *Trichoderma* and plant interactions

The genus *Trichoderma* is one of the most widely researched genera of filamentous fungi with numerous applications in agriculture, industry and the environment (Fig. 2) (Schuster and Schmol, 2010; Mukherjee et al., 2013). Several *Trichoderma* species are used extensively for the production of industrial enzymes and there is hope that their powerful biodegradative enzymes can be employed for biofuel production. In particular, *T. reesei* is grown industrially for the production of cellulolytic and hemicellulolytic enzymes. Several species are used in the bioremediation of wastes including metals in soil (Bishnoi et al., 2007; Morales-Barrera and Cristiani-Urbina, 2008; Tripathi et al., 2013). *Trichoderma* species are known producers of secondary metabolites with medical and agricultural significances as they often exhibit anticancer, antifungal, antibacterial, and toxic properties (Mathivanan et al., 2008; Mukherjee et al., 2013). *Trichoderma* species are robust biological control agents because they utilize several modes of action including resistance, antibiosis, competition and myco-parasitism (Whipps and Lumsden, 1989). *Trichoderma* is commonly used as biological control agents, especially in less developed countries. Since *Trichoderma* possess innate resistance to many chemicals used in agriculture such as fungicides, they are readily used as part of the integrated pest management practices (Chaparro et al., 2011).

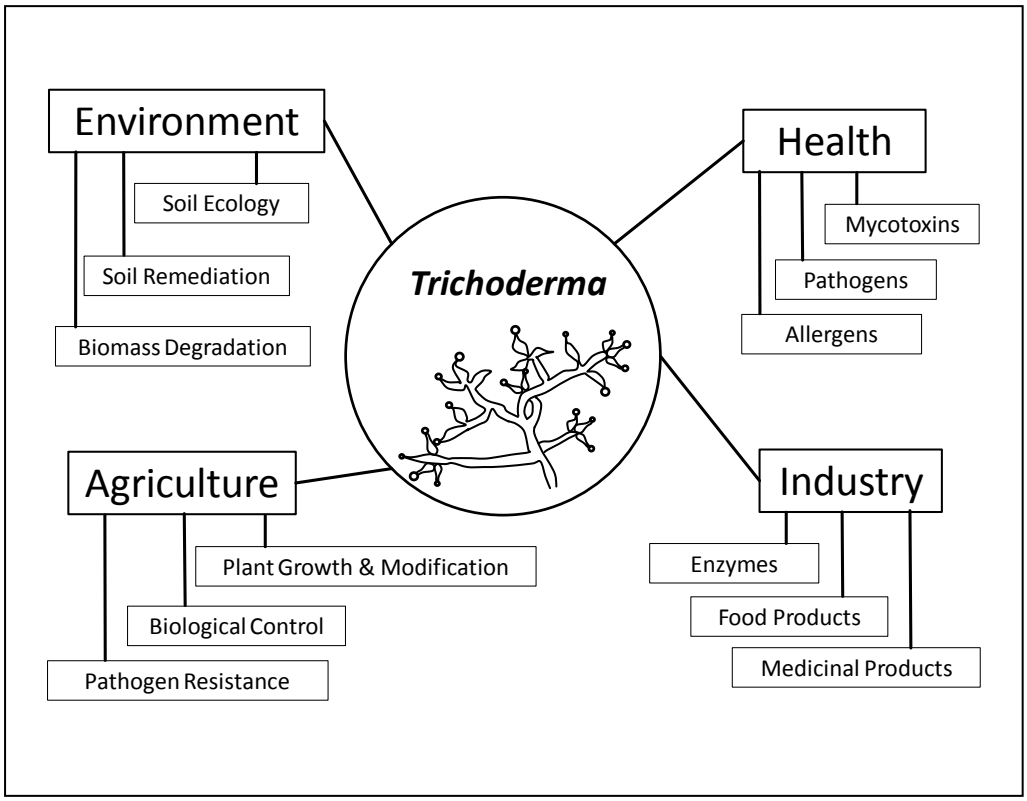

Figure 2. Application of *Trichoderma* and major areas of research.

Trichoderma species are found in nearly all temperate and tropical soils. They readily colonize woody and herbaceous plant materials and are common in the rhizosphere. While the association between plants and *Trichoderma* is classified as symbiotic they have the ability to reduce plant diseases and promote plant growth and productivity (Harman et al., 2004; Ortiz-Castro et al., 2009; Szabo et al., 2012). *Trichoderma* spp. found in close association with plant roots have many direct and

indirect effects including the ability to produce antibiotic substances (antibiosis), parasitize other fungi and nematodes (mycoparasitism), and compete successfully against other microorganisms (competitive and antagonistic potentials). These diverse mechanisms of plant improvement by *Trichoderma* are dependent on species and environmental conditions (Mukherjee et al., 2013).

Trichoderma species are also known to aid in nutrient uptake, provide efficient nitrogen usage, and solubilize nutrients in the soil under sub-optimal conditions (Harman et al., 2004; Mastouri et al., 2010; Shoresh et al., 2010). The colonization of plant roots by *Trichoderma* enhances photosynthetic abilities and induces defense responses (Vargas et al., 2009). Abiotic stresses such as drought and salinity, as well as physiological stresses such as seed dormancy, aging, and oxidative stress, can be alleviated by the fungi (Mastouri et al., 2010; 2012; Delgado-Sánchez et al., 2011). Marine isolates of *Trichoderma* have been applied as biological control agents in saline and arid soils (Gal-Hemed et al., 2011). Identification of novel secondary metabolite production by endophytic *Trichoderma* to reduce biotic and abiotic stress is emerging areas of study (Bae et al., 2011).

Furthermore, certain species of *Trichoderma* colonize roots and induce local and systemic resistance in plants via the production of elicitors. Elicitors, such as *small protein 1*, trigger the production of reactive oxygen species and ultimately the expression of defense-related genes (Harman et al., 2004; Djonovic et al., 2006). Jasmonate, salicylate, and ethylene signaling pathways are activated by *Trichoderma* species and have been implicated in systemic resistance in plants (Lorito et al., 2010; Bae et al., 2011; Hermosa et al., 2012). In addition, species such as *T. virens* and *T. atroviride* produce the

phytohormone, indole-3-acetic acid (IAA) and other auxin-related compounds. These compounds cause an increase in abscisic acid biosynthesis resulting in plant development and immune responses (Contreras-Cornejo et al., 2009; Mastouri et al., 2010).

1.10 The production of VOCs by *Trichoderma* and biological activities

Trichoderma species are prolific producers of VOCs (Siddiquee et al., 2012; Mukherjee et al., 2013). One of the earliest volatile compounds to be identified from *Trichoderma* was the coconut odor, 6-pentyl-2H-pyran-2-one (6PP) (Collins and Halim, 1972). Although the main use of 6PP has been as a food additive, treating plants with a low concentration of 6PP (0.166 - 1 mg/l) induced growth promotion and reduced disease symptoms (Vinale et al., 2008).

Trichoderma species are prolific producers of many other volatiles, in particular pyrones and sesquiterpenes. Some *Trichoderma* volatiles can be self inhibitory, inhibit other fungi, or induce sexual mating in certain species, leading to the suggestion that the volatile themselves might have applications as biological control agents (Brasier et al., 1993; Bruce et al., 2000; Aguero et al., 2008). In the following paragraphs, volatile production and volatile-mediated effects of some of the most important *Trichoderma* species used in agriculture and environmental studies are summarized.

T. harzianum is widely used in agriculture for biological control. As an active root colonizer, *T. harzianum* improves mineral uptake and solubilization, induces systemic resistance, and alleviates abiotic and physiological stress in plants (Harman et al., 2004; Mastouri et al., 2010; Hermosa et al., 2012). *T. harzianum* produces common volatiles such as ethanol, 3-methyl-1-butanol, 2-methyl-1-butanol, and 3-octanone, as well as a range of sesquiterpenes (Fiedler et al., 2001; Citron et al., 2011). When grown

on potato dextrose agar (PDA), Siddiquee et al., (2012) detected 278 volatile compounds from *T. harzianum* ranging in size from C7 to C30, including cyclohexane, cyclopentane, fatty acids, alcohols, esters, sulfur-containing compounds, pyrans, and benzene derivatives.

T. harzianum also produces pyrone-like volatile metabolites that suppress the growth of the plant pathogen, *Gaeumannomyces graminis* (Ghisalberti et al., 1990). Furthermore, *T. harzianum* VOCs inhibit the growth and accumulation of aflatoxin production by *Aspergillus flavus,* and have been proposed as a biological control strategy for the inhibition of mycotoxin production during crop storage (Aguero et al., 2008). Volatile emissions of the C8 compounds 1-octen-3-ol, 3-octanol, and 3-octanone of *T. harzianum* elicit conidiation in neighboring colonies (Nemcovic et al., 2008). The conidiation-inducing effect could be replicated using chemical standards of the C8 compounds and the effects were concentration dependent with higher concentrations being inhibitory. In other studies, 1-octen-3-ol also was found to be a self inhibitor of germination and an inducer of micro-conidiation in mycotoxin-producing *Penicillium* (Chitarra et al., 2004).

T. viride is another species commonly used in biological control. It improves mineral uptake and solubilization, produces cytokinin-like compounds, and is able to elicit jasmonic acid, salicylic acid, and ethylene biosynthesis in plants (Hermosa et al., 2012). In addition to 6PP it produces several unique sesquiterpenes such as γ-curcumene, α-zingiberene, and tricho-acorenol (Lloyd et al., 2005). Detailed biosynthesis of tricho-acorenol and other sesquiterpene production by *T. viride*, *T. harzianum*, and *T. longibrachiatum* are available (Citron et al., 2011; Citron and Dickschat, 2013). More

than 22 volatile compounds have been identified from *T. viride*, with 2-propanone, 2-methyl-1-butanol, and heptanal shown to be important in inhibiting wood decay fungi (Wheatley et al., 1997). Similar inhibition of wood decay fungi was observed in a separate study 2-methyl-1-butanol, heptanal, and octanal (Humphris et al., 2001). The inhibition of growth rate was correlated with an inhibition of protein synthesis in the presence of the *T. viride* VOC mixture. Wood decay fungi resumed growth and protein synthesis when the *Trichoderma* culture was removed from the exposure conditions (Humphris et al., 2002). In another study, *T. viride* VOCs promoted all aspects of plant growth including plant size, fresh weight, root growth, increase chlorophyll, and larger number of flowers. An analysis of this growth-promoting mixture of VOCs by GC-MS gave 51 compounds, of which 2-methyl-1-propanol, 3-methyl-1-butanol, and 3-methylbutanal were most abundant (Hung et al., 2013).

T. atroviride VOCs are able to a induce defense response in plants and are known plant growth regulators through mechanisms such as indole acetic acid (IAA) and ethylene regulation (Gravel et al., 2007). In addition to producing 6PP, *T. atroviride* produced four derivatives of pyrone, 6-n-butanal-2H-pyran-2-one, 5,5-dimethyl-2H-pyran-2-one, and 6-n-pent-1,2-enyl-2H-pyran-2-one (Keszler et al., 2000). On PDA it emitted up to 25 compounds, including several monoterpenes and sesquiterpenes (Stoppacher et al., 2010). Further analysis of terpene production showed that it was time dependent, where sesquiterpenes were produced after monoterpenes, and where C8 compounds such as 1-octen-3-ol and 3-octanone were produced still later, following sporulation (Polizzi et al., 2012). A large number of compounds were produced when the fungi was grown on MEA. However, changing the substrate from fungal media to

building materials (wallpaper and plasterboard) caused production of new compounds such as 2-ethyl-cyclopentatnone, menthone, and 4-heptanone. Exposure to *T. atroviride* VOCs affects conidiation in fungi and inhibits wood decay fungi (Steyaert et al., 2010).

When used synergistically with arbuscular mycorrhizal (AM) fungi, *T. aureoviride* increases plant biomass (Calvert et al., 1993). Changing the amino acid composition of the growth medium altered the VOC production in *T. aureoviride* yielding a total of 30 VOCs. The levels of inhibition of the wood decay fungi were dependent on the C:N ratio of the substrate, and the production of aldehydes and ketones was associated with the greatest inhibition (Bruce et al., 2000). Similar to *T. viride*, *T. aureoviride* VOCs have the ability to inhibit mycelial growth and protein synthesis in wood decay fungi (Humphris et al., 2002).

T. pseudokoningii induces a defense response and systemic resistance in tobacco against tobacco mosaic virus infection (Luo et al., 2010). The fungal production of non-volatile metabolite, peptaibols (Trichokonins) enhances pathogenesis-related reactive oxygen species and phenolic compounds, and induces defense genes in plants. *T. pseudokoningii* also produces volatile antibiotic compounds that inhibit spore germination in AM fungi (Martinez et al., 2004) and several wood decay fungi (Wheatley et al., 1997).

Volatile profiles of *T. viride* and *T. pseudokoningii* grown MEA and minimal media were compared (Wheatley et al., 1997). A total of 45 VOCs were identified; the production of individual compounds was dependent on the species of *Trichoderma* and the growth media type. The VOC-induced inhibition of wood decay fungi was the greatest from fungi grown on MEA. When the fungi were grown on a minimal medium,

the effects were negligible. When the volatile profiles of *T. harzianum* and *T. pseudokoningii* growing on MEA were compared, a specific pattern of sesquiterpenes and other compounds were found (Fiedler et al., 2001).

Antibiosis of *T. koningii* includes broad-spectum antimicrobial activity through Trichokonins production and inhibition of plant pathogen growth in the rhizosphere making this fungi an ideal biological control (Tsahouridou and Thanassoulopoulos, 2002; Xiao-Yan et al., 2006). The antibiotic effect also has a negative effect in that it decreases the growth of AM fungi in the rhizosphere (McAllister et al., 1994). When *T. koningii* directly interacts with plants, it increases seedling formation, fresh weight, and the height of plants. Detection of the volatiles 1,3-pentadiene and styrene, have been implicated in food spoilage by *T. koningii* (Pinches and Apps, 2008). *T. koningii* does not produce 6PP when grown on PDA; however, it produces 22 other compounds including sesquiterpenes (Jelen et al., 2014). When VOCs from several species of *Trichoderma* VOCs were compared, 1-octen-3-ol, isoamyl alcohol, 3-octanone, cyclohept-3-en-1-one, 2-pentylfuran, linalyl isobutyrate, toluene, limonene, and α-bergamotene were commonly found. In summary, *Trichoderma* VOCs are potent, biologically active compounds with the potential for numerous applications in agriculture. However, the mechanisms involved in volatile-mediated effects are yet to be determined (Zeilinger and Schumacher, 2013).

1.11 Concluding remarks

Although VOCs from fungi have been studied for decades in chemotaxonomy, in food and flavor chemistry, as semiochemicals for insects, and as indirect indicators of fungal growth, the recognition of the role of VOCs in plant microbial ecology is

relatively new (Morath et al., 2012; Bitas et al., 2013; Hung et al., 2015). Fungi interact with organisms that share the same habitat, maintaining the ecological and functional balance of the soil. While there is a high diversity in volatile production, VOCs can be used as a noninvasive indicators of microbial communities in soil. It is important to note that the documented VOC profiles do not necessarily reflect the complete picture of the volatile emission in the environment. Our increasing knowledge of volatile production, understanding the effects of different environmental conditions and microorganism specific conditions, will provide more understanding of the structure, physiological state, and activity of microbial communities in soil.

In agriculture, *Trichoderma* species have been commercialized as plant growth promoters and for protection against pathogens. They have the ability to thrive in wide range of soil conditions and substrates and have resistance to chemicals, making them ideal biological control agents. Some *Trichoderma* species have been analyzed for their volatile productions and hundreds of volatile compounds have been identified; notably including pyrones and sesquiterpenes. Select volatiles are involved in signaling and communication in microbial communities and can suppress the growth of soil organisms. There is increasing evidence that some *Trichoderma* volatiles are bio-stimulatory and have the potential to enhance plant growth and development, including tolerance to biotic and abiotic stresses and induction of plant resistance to pathogens. To date, VOC profiles have been conducted only on a few *Trichoderma* isolates. Because these studies were conducted with different species, differing in age of the culture, growth media, VOC analytical methods, they are not easy to compare. Moreover, the biological activities have been focused on *Trichoderma*-to-fungi interactions. The exploitation of

Trichoderma volatiles as plant growth promoters and/or disease suppressors has the potential to become a powerful tool in agriculture. A critical step in the improved practical application of these VOCs is to develop a mechanistic understanding of the volatile-mediated *Trichoderma*-to-plant interactions.

1.12 Overview of the research goals presented in this study

The overarching goal of this research is to develop a mechanistic understanding of the volatile-mediated *Trichoderma*-to-plant interactions. The research presented in this dissertation has used *Arabidopsis thaliana* as a model to study volatile-mediated interactions between *Trichoderma* and plants. The first objective was to develop a relatively quick and reproducible exposure method to study volatile-mediated effects; and to determine the physiological and environmental parameters that affect fungal volatile production and in turn their effects in plants. The second objective was to complete a large screening of different strains and species of *Trichoderma* with the goal of finding isolates that can produce optimal plant growth promoting volatiles. The biological screening was accompanied by analysis of the *Trichoderma* volatile emission profiles, concentrations, and quantities detected through GC-MS. The third objective was to study a subgroup of VOCs identified in the GC-MS analysis and expose *Arabidopsis* to chemical standards of these individual compounds in order to identify specific compounds responsible for plant growth promotion. The fourth objective was to select a representative, growth-promoting fungal VOC and complete a global gene expression study using RNA sequencing to identify potential candidate genes associated with volatile-mediated responses in *Arabidopsis*. Finally, using the standardized and controlled methods developed in this dissertation research, VOCs from an entirely

different genus of filamentous fungi were tested. Two strains of *Aspergillus versicolor* were compared for the effect of substrate composition on volatile production and plant responses to blends and chemical standards of individual *Aspergillus* volatiles.

REFERENCES

Aguero, L.E.M., Alvarado, R., Martinez, A., Dorta, B. (2008). Inhibition of *Aspergillus flavus* growth and aflotoxin B1 production in stored maize grains exposed to volatile compounds of *Trichoderma harzianum* rifai. Interciencia. 33: 219-222.

Alpha, C.J., Campos, M., Jacobs-Wagner, C., Strobel, S.A. (2015). Mycofumigation by the volatile organic compound-producing gungus *Muscodor albus* induces bacterial cell death through DNA damage. Applied Environmental Microbiology. 81: 1147-1156.

Aspray, T.J., Jones, E.E., Whipps, J.M., Bending, G.D. (2006). Importance of mycorrhization helper bacteria cell density and metabolite localization for the *Pinus sylvestris-Lactarius rufus* symbiosis. FEMS Microbiology Ecology. 56: 25-33.

Asensio, D., Penuelas, J., Filella, I., Llusià, J. (2007). On-line screening of soil VOCs exchange responses to moisture, temperature and root presence. Plant Soil. 291: 294-261.

Atmosukarto, I., Castillo, U., Hess, W.M., Sears, J., Strobel, G. (2005). Isolation and characterization of *Muscodor albus* I-41.3s, a volatile antibiotic producing fungus. Plant Science. 169: 854-861.

Bae, H., Roberts, D.P., Lim, H.S., Strem, M.D., Par, S.C., Ryu, C.M., Melnick, R.L., Bailey, B.A. (2011). Endophytic *Trichoderma* isolates from tropical environments delay disease onset and induce resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms. Molecular Plant-Microbe Interactions Journal. 24: 336-351.

Bagheri, H.R., Moghadam, A.R.L., Afshari, H. (2014). The effects of foliar application of methanol on growth and secondary metabolites in lavender. International Research Journal of Applied and Basic Sciences. 8: 150-152.

Bailey, K.L., Boyetchko, S.M., Längle, T. (2010). Social and economic drivers shaping the future of biological control: a Canadian perspective on the factors affecting the development and use of microbial biopesticides. Biological Control. 52: 221-219.

Banerjee, D., Strobel, G.A., Booth, E., Geary, B., Sears, J., Spakowicz, D., Busse, S. (2010). An endophytic *Myrothecium inundatum* producing volatile organic compounds. Mycosphere. 229-240.

Basheer, C., Valiyaveettil, S., Lee, H.K. (2010). Recent trends in sample preparation for pesticide analysis. In: Nollet, L.M.L., Rathore, H.S. (Ed.) Handbook of pesticides: methods of pesticide residues analysis. CRC Press. Taylor & Francis Group. Pgs. 381-394.

Betancourt, D.A., Krebs, K., Moore, S.A., Martin, S.M. (2013). Microbial volatile organic compound emissions from *Stachybotrys chartarum* growing on gypsum wallboard and ceiling tile. BMC Microbiology. 13: 283-293.

Bishnoi, N.R., Kumar, R., Bishnoi, K. (2007). Biosorption of Cr (VI) with *Trichoderma viride* immobilized fungal biomass and cell free Ca-alginate beads. Indian Journal of Experimental Biology. 45: 657-664.

Bitas, V., Kim, H.-S., Bennett, J., Kang, S. (2013). Sniffing on microbes: diverse roles of microbial volatile organic compounds in plant health. Molecular Plant-Microbe Interactions Journal. 4: 835-843.

Blom, D., Fabbri, C., Connor, E.C., Schiestl, F.P., Klauser, D.R., Boller, T., Eberl, L., Weisskopf, L. (2011). Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. Environmental Microbiology. 13: 3047-3058.

Bonfante, P., Anca, I.A. (2009). Plants, mycorrhizal fungi, and bacteria: a network of interactions. Annual Review of Microbiology. 63: 363-383.

Brasier, C.M., Hamm, P.B., Hansen, E.M. (1993). Cultural characters, protein patterns and unusual mating behavior of *Phytophthora gonapodyides* isolates from Britain and North America. Mycological Research. 97: 1287-1298.

Bruce, A., Stewart, D., Verrall, S., Wheatley, R.E. (2003). Effect of volatiles from bacteria and yeast on the growth and pigmentation of sapstain fungi. International Biodeterioation & Biodegradation. 51: 101-108.

Bruce, A., Wheatley, R.E., Humphris, S.N., Hackett, C.A., Florence, M.E.J. (2000). Production of volatile organic compounds by *Trichoderma* in media containing different amino acids and their effect on selected wood decay fungi. Holzforschung. 54: 481-486.

Burmølle, M., Hansen, L.H., Sørensen, S.J. (2007). Establishment and early succession of a multispecies biofilm composed of soil bacteria. Microbial Ecology. 54: 352-362.

Butt, T.M., Copping, L.G. (2000). Fungal biological control agents. Pesticide Outlook. 11: 186-191.

Calvert, C., Pera, J., Barea, J.M. (1993). Growth response of marigold (*Tagetes erecta* L.) to inoculation with *Glomus mosseae*, *Trichoderma aureoviride* and *Pythium ultimum* in a peat-perlite mixture. Plant and Soil. 148: 1-6.

Cape, J.N. (2003). Effects of airborne volatile organic compounds on plants. Environmental Pollution. 122: 145-157.

Cardoza, Y., Alborn, H.T., Tumlinson, J.H. (2002). In vivo volatile emissions from peanut plants induced by simultaneous fungal infection and insect damage. Journal of Chemical Ecology. 28: 161- 174.

Chakraborty, M.R., Chatterjee, N.C. (2008). Control of fusarium wilt of *Solanum melongena* by *Trichoderma* spp. Biologia Plantarum. 52: 582-586.

Chaparro, A.P., Carvajal, L.H., Orduz, S. (2011). Fungicide tolerance of *Trichoderma sperelloides* and *T. harzianum* strains. Agricultural Sciences. 2: 301-307.

Chaurasia, B., Pandey, A., Palni, L.M.S., Trivedi, P., Kumar, B., Colvin, N. (2005). Diffusible and volatile compounds produced by an antagonistic *Bacillus subtilis* strain

cause structural deformations in pathogenic fungi in vitro. Microbiological Research. 160: 75-81.

Chitarra, G.S., Abee, T., Rombouts, F.M., Posthumus, M.A., Dijksterhuis, J. (2004). Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. Applied Environmental Microbiology. 70: 2823-2829.

Chuankun, X., Minghe, M., Leming, Z., Keqin, Z. (2004). Soil volatile fungistasis and volatile fungistatic compounds. Soil Biology and Biochemistry. 36: 1997-2004.

Citron C.A., Dickschat, J.S. (2013). The stereochemical course of tricho-acorenol biosynthesis. Organic & Biomolecular Chemistry. 11: 7447-7450.

Citron, C.A., Riclea, R., Brock, N.L., Dickschat, J.S. (2011). Biosynthesis of acorane sesquiterpenes by *Trichoderma*. RSC Advances. 1: 290-297.

Collins, R.P., Halim, A.F. (1972). Characterization of the major aroma constituent of the fungus *Trichoderma viride* (Pers.). Journal of Agricultural and Food Chemistry. 20: 437-738.

Comandini, A., Malewicki, T., Brezinsky, K. (2012). Online and offline experimental techniques for polycyclic aromatic hydrocarbons recovery and measurement. Review of Scientific Instruments (Rev. Sci. Instrum.). 83: 034101. doi:10.1063/1.3692748.

Combet, E., Henderson, J., Eastwood, D.C., Burton, K.S. (2006). Eight carbon volatiles in fungi: properties, analysis, and biosynthesis. Mycoscience. 47: 317-326.

Contreras-Cornejo, H.A., Macias-Rodriguez, L.I., Cortes-Penagos, C., Lopez-Bucio, J. (2009). *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. Plant Physiology. 149: 1579-1592.

Crespo, E., Hordijk, C.A., de Graaf, R.M., Samudrala, D., Cristescu, S.M., Harren, F.J.M., Dam, N.M. (2012). On-line detection of root-induced volatiles in *Brassica nigra* plants infested with *Delia radicum* L. root fly larvae. Phytochemistry. 84: 68-77.

Dandurishvili, N., Toklikishvili, N., Ovadis, M., Eliashvili, P., Giorgobiani, N., Keselava, R., Tediashvili, M., Vainstein, A., Khmel, I., Szegedi, E., Chernin, L. (2010). Broad-range antagonistic rhizobacteria *Pseudomonas fluorescens* and *Serratia plymuthica* suppress *Agrobacterium* crown gall tumors on tomato plants. Journal of Applied Microbiology. 110: 341-352.

Davis, T.S., Crippen, T.L., Hofstetter, R.W., Tomberlin, J.K. (2013). Microbial volatile emissions as insect semiochemicals. Journal of Chemical Ecology. 4: 840-859.

Delgado-Sánchez, P., Ortega-Amaro, M.A., Jiménez-Bremont, J.F., Flores, J. (2011). Are fungi important for breaking seed dormancy in desert species? Experimental evidence in *Opuntia streptacantha* (Cactaceae). Plant Biology. 13: 154-159.

Del Giudice, L., Massardo, D.R., Pontieri, P., Bertea, C.M., Mombello, D., Carata, E., Tredici, S.M., Tala, A., Mucciare, M., Groudeva, V.I., De Stefano, M., Vigliotta, G., Maffei, M.E., Alifano, P. (2008). The microbial community of Vetiver root and its involvement into essential oil biogenesis. Environmental Microbiology. 10: 2824-2841.

Dennis, C., Webster, J. (1971). Antagonistic properties of species-groups of *Trichoderma* 11. Production of volatile antibiotics. Transactions of the British Mycological Society. 57: 41-48.

Djonovic, S., Pozo, M.J., Dangott, L.J., Howell, C.R., Kenerley, C.M. (2006). *Sm1*, a proteinaceous elicitor secreted by the biocontrol fungus *Trichoderma virens* induces plant defense responses and systemic resistance. Molecular Plant-Microbe Interactions Journal. 19: 838-853.

Dudareva, N., Klempien, A., Muhlemann, J.K., Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. New Phytologist. 198: 16-32.

Effmert, U., Kalderas, J., Warnke, R., Piechulla, B. (2012). Volatile mediated interactions between bacteria and fungi in the soil. Journal of Chemical Ecology. 38: 665-703.

Ezra, D., Hess, W.M., Strobel, G.A. (2004). New endophytic isolates of *Muscodor albus*, a volatile-antibiotic-producing fungus. Microbiology. 150: 4023-4031.

Farag, M.A., Fokar, M., Abd, H., Zhang, H., Allen, R.D., Pare, P.W. (2005). (Z)-3-Hexenol induces defense genes and downstream metabolites in maize. Planta. 220: 900-909.

Farag, M.A., Zhang, H., Ryu, C.M. (2013). Dynamic chemical communication between plants and bacteria through airborne signals: induced resistance by bacterial volatiles. Journal of Chemical Ecology. 39: 1007-1018.

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C. (2005). Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biology & Biochemistry. 37: 955-964.

Ferry, A., Dugravot, S., Delattre, T., Christides, J.P., Auger, J., Bagneres, A.G., Poinsot, D., Cortesero, A.M. (2007). Identification of a widespread monomolecular odor differentially attractive to several *Delia radicum* ground-dwelling predators in the field. Journal of Chemical Ecology. 33: 2064-2077.

Fiddaman, P.J., Rossall, S. (1993). The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology. 74: 119-126.

Fiedler, K., Schutz, E., Geh, S. (2001). Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. International Journal of Hygiene Environmental Health. 204: 111-121.

Fiers, M., Lognay, G., Fauconnier, M.-L., Jijakli, M.H. (2013). Volatile compound-mediated interactions between barley and pathogenic fungi in the soil. PLoS ONE. 8(6): e66805. doi:10.1371/journal.pone.0066805.

Frost, C.J., Appel, H.M., Carlson, J.E., De Moraes, C.M., Mescher, M.C., Schultz, J.C. (2007). Within-plant signaling via volatiles overcomes vascular constraints on systemic signaling and primes responses against herbivores. Ecology Letters. 10: 490-498.

Galbally, I.E., Kirstine, W. (2002). The production of methanol by flowering plants and the global cycle of methanol. Journal of Atmospheric Chemistry. 43: 195-229.

Gal-Hemed, I., Atanasova, L., Komon-Zelazowska, M., Druzhinina, I.S., Viterbo, A., Yarden, O. (2011). Marine isolates of *Trichoderma* spp. as potential halotolerant agents of biological control for arid-zone agriculture. Applied and Environmental Microbiology. 77: 5100-5109.

Gardener, B.B.M., Fravel, D.R. (2002). Biological control of plant pathogens: research, commercialization, and application in the USA. Plant Health Progress. doi:10.1094/PHP-2002-0510-01-RV.

Ghisalberti, E.L., Narbey, M.J., Dwan, J.M., Sivasithamparam, K. (1990). Variability among strains of *Trichoderma harzianum* in their ability to reduce take-all and to produce pyrones. Plant and Soil. 121: 287-291.

Gravel, V., Antoun, H., Tweddell, R.J. (2007). Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonasputida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). Soil Biology & Biochemistry. 39: 1968-1977.

Greenberg, J.P., Asensio, D., Turnipseed, A., Guenther, A.B., Karl, T., Gochis, D. (2012). Contribution of leaf and needle litter to whole ecosystem BVOC fluxes. Atmospheric Environment. 59: 302-311.

Groen, S.C., Whiteman, N.K. (2014). The evolution of ethylene signaling in plant chemical ecology. Journal of Chemical Ecology. 40: 700-716.

Gutiérrez-Luna, F.M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., de la Cruz, H.R., Macías-Rodríguez, L. (2010). Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. Symbiosis. 51: 75-83.

Hamamoto, S., Moldrup, P., Kawamoto, K., Komatsu, T. (2012). Organic matter fraction dependent model for predicting the gas diffusion coefficient in variably saturated soils. Vadose Zone Journal. doi: 10.2136/vzj2011.0065.

Hann, C.T., Bequette, C.J., Dombrowski, J.E., Stratmann, J.W. (2014). Methanol and ethanol modulate responses to danger- and microbe-associated molecular patterns. Frontiers in Plant Science. 5: 550. doi: 10.3389/fpls.2014.00550.

Hansel, A., Jordan, A., Holzinger, R., Prazeller, P., Vogel, W., Lindinger, W. (1995). Proton transfer reaction mass spectrometry: on-line trace gas analysis at the ppb level. International Journal of Mass Spectrometry. 149: 609-619.

Hanson, R. (1992). Methane and methanol utilizers. In: Colin Murrell, J., Dalton, H. (Ed.) Biotechnology Handbooks 5. Springer Science+Business Media. Pgs. 1-15.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M. (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. Nature Reviews Microbiology. 2: 43-56.

Heddergott, C., Calvo, A.M., Latge, J.P. (2014). The volatome of *Aspergillus fumigatus*. Eukarotic Cell. 13: 1014-1025.

Heil, M., Silva Bueno, J.C. (2007). Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. Proceedings of the National Academy of Sciences. 104: 5467-5472.

Hermosa, G., Viterbo, A., Chet, I., Monte, E. (2012). Plant-beneficial effects of *Trichoderma* and of its genes. Microbiology. 158: 17-25.

Hinton Jr., A., Hume, M.E. (1995). Antibacterial activity of the metabolic by-products of a *Veillonella* species and *Bacteroides fragilis*. Anaerobe. 1: 121-127.

Huang, C.J., Tsay, J.F., Chang, S.Y., Yang, H.P., Wu, W.S., Chen, C.Y. (2012). Dimethyl disulfide is an induced systemic resistance elicitor produced by *Bacillus cereus* C1L. Pest Management Science. 68: 1306-1310.

Hubschmann, H.-J. (2015). Handbook of GC-MS. Fundamentals and applications. 3rd edition. John Wiley & Sons.

Hueve, K., Christ, M.M., Kleist, E., Uerlings, R., Niinemets, U.,Walter, A., Wildt, J. (2007). Simultaneous growth and emission measurements demonstrate an interactive control of methanol release by leaf expansion and stomata. Journal of Experimental Botany. 58: 1783-1793.

Humphris, S.N., Bruce, A., Buultjens, E., Wheatley, R.E. (2002). The effects of volatile microbial secondary metabolites on protein synthesis in *Serpula lacrymans*. FEMS Microbiology Letters. 210: 215-219.

Humphris, S.N., Wheatley, R.E., Bruce, A. (2001). The effects of specific volatile organic compounds produced by *Trichoderma* spp. on the growth of wood decay basidiomycetes. Holzforschung. 55: 233-237.

Hung, R., Lee, S., Bennett, J.W. (2013). *Arabidopsis thaliana* as a model system for testing the effects of *Trichoderma* volatile organic compounds. Fungal Ecology. 6: 19-26.

Hung, R., Lee, S., Bennett, J.W. (2014). The effects of low concentrations of the enantiomers of mushroom alcohol (1-octen-3-ol) on *Arabidopsis thaliana*. Mycology: An International Journal of Fungal Biology. 5: 73-80.

Hung, R., Lee, S., Bennett, J.W. (2015). Fungal volatile organic compounds and their role in ecosystems. Applied Microbiology and Biotechnology. 99: 3395-3405.

Hynes, J., Müller, C.T., Jones, T.H., Boddy, L. (2007). Changes in volatile production during the course of fungal mycelial interactions between *Hypholoma fasciculare* and *Resinicium bicolor*. Journal of Chemical Ecology. 33: 43-57.

Iguchi, H., Yurimoto, H., Yasuyoshi, S. (2015). Interactions of methylotrophs with plants and other heterotrophic bacteria. Microorganisms. 3: 137-151.

Insam, H., Seewald, S.A. (2010). Volatile organic compounds (VOCs) in soils. Biology and Fertility of Soils. 46: 199-213.

Isidorov, V., Jdanova, M. (2002). Volatile organic compounds from leaves litter. Chemosphere. 48: 975-979.

Jassbi, A.R., Zamanizadehnajari, S., Baldwin, I.T. (2010). Phytotoxic volatiles in the roots and shoots of *Artemisia tridentata* as detected by headspace solid-phase microextraction and gas chromatographic-mass spectrometry analysis. Journal of Chemical Ecology. 36: 1398-1407.

Jelen, H., Blaszczyk, L., Jerzy, C., Rogowicz, K., Strakowska, J. (2014). Formation of 6-n-pentyl-2H-pyran-2-one (6-PAP) and other volatiles by different *Trichoderma* species. Mycological Progress. 13: 589-600.

Kai, M., Crespo, E., Cristescu, S.M., Harren, F.J.M., Piechulla, B. (2010). *Serratia odorifera*: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*. Applied Microbiology and Biotechnology. 88: 965-976.

Kai, M., Effmert, U., Ber, G., Piechulla, B. (2007). Volatiles of bacterial antagonists inhibit mycelial growth of the plant pathogen *Rhizoctonia solani*. Archives of Microbiology. 187: 351-360.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., Piechulla, B. (2009). Bacterial volatiles and their action potential. Applied Microbiology and Biotechnology. 81: 1001-1012.

Karlshoj, K., Nielsen, P.V., Larsen, T.O. (2007). Fungal volatiles: biomarkers of good and bad food quality. In: Samson, R.A., Dijksterhus, J., Boca Raton, F.L. (Ed.) Food Mycology. CRC Press. Pgs. 279-302.

Ketola, R.A., Kiuru, J.T., Kotiaho, T., Kitunen, V., Smolander, A. (2011). Feasibility of membrane inlet mass spectrometry for on-site screening of volatile monoterpenes and monoterpene alcohols in forest soil atmosphere. Boreal Environment Research. 16: 36-46.

Keszler, A., Forgacs, E., Kotai, L., Vizcaino, J.A., Monte, E., Garcia-Acha, I. (2000). Separation and identification of volatile components in the fermentation broth of *Trichoderma atroviride* by solid-phase extraction and gas chromatography-mass spectrometry. Journal of Chromatographic Science. 38: 421-424.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J. (2007). Volatile 1-octen-3-ol induces a defensive response in *Arabidopsis thaliana*. Journal of Plant Pathology. 73: 35-37.

Kolb, S. (2009). Aerobic methanol-oxidizing *Bacteria* in soil. FEMS Microbiology Letters. 300: 1-10.

Korpi, A., Jarnberg, J., Pasanen, A.L. (2009). Microbial volatile organic compounds. Critical Reviews in Toxicology. 39: 139-193.

Kudalkar, P., Strobel, G., Hasan, S.R.U., Geary, G., Sears, J. (2012). *Muscodor sutura* a novel endophytic fungus with volatile antibiotic activities. Mycoscience. 53: 319-325.

Larsen, T.O., Frisvad, J.C. (1995a). Characterization of volatile metabolites from 47 *Penicillium* taxa. Mycological Research. 99: 1153-1166.

Larsen, T.O., Frisvad, J.C. (1995b). Comparison of different methods for collection of volatile chemical markers from fungi. Journal of Microbiological Methods. 24: 135-144.

Lee, S., Hung, R., Schink, A., Mauro, J., Bennett, J.W. (2014). Phytotoxicity of volatile organic compounds. Plant Growth Regulation. 74: 177-186.

Lee, S., Hung, R., Yap, M., Bennett, J.W. (2015a). Age matters: the effects of volatile organic compounds emitted by *Trichoderma atroviride* on plant growth. Archives of Microbiology. 197: 723-727.

Lee, S., Hung, R., Yin, G., Klich, M.A., Grimm, C., Bennett, J.W. (2015b). Strain specific interaction between *Aspergillus versicolor* and plants through volatile organic compounds. *Under Review.*

Lee, S.O., Kim, H.Y., Choi, G.J., Lee, H.B., Jang, K.S., Choi, Y.H., Kim, J.C. (2009). Mycofumigation with *Oxyporus latemarginatus* EF069 for control of postharvest apple decay and *Rhizoctonia* root rot on moth orchid. Journal of Applied Microbiology. 106: 1213-1219.

Leff, J.W., Fierer, N. (2008). Volatile organic compound (VOC) emissions from soil and litter samples. Soil Biology & Biochemistry. 40: 1629-1636.

Lemfack, M.C., Nickel, J., Dunkel, M., Preissner, R., Piechulla, B. (2014). VOC: a database of microbial volatiles. Nucleic Acids Research. 42: 744-748.

Linton, C.J., Wright, S.J.L. (1993). Volatile organic compounds: microbiological aspects and some technological implications. Journal of Applied Bacteriology. 75: 1-12.

Lloyd, D., Thomas, K.L., Cowie, G., Tammam, J.D., Williams, A.G. (2002). Direct interface of chemistry to microbiological systems: membrane inlet mass spectrometry. Journal of Microbiological Methods. 48: 289-302.

Lloyd, W.W., Grimm, C., Klich, M., Beltz, S.B. (2005). Fungal infections of fresh-cut fruit can be detected by the gas chromatography-mass spectrometric identification of microbial volatile organic compounds. Journal of Food Protection. 68: 1211-1216.

Lorito, M., Woo, S.L., Harman, G.E., Monte, E. (2010). Translational research on *Trichoderma*: from 'omics to the field. Annual Review of Phytopathology. 48: 395-417.

Luo, Y., Zhang, D.D., Dong, X.W., Zhao, P.B., Chen, L.L., Song, X.Y., Wang, X.J., Chen, X.L., Shi, M., Zhang, Y.Z. (2010). Antimicrobial peptaibols induce defense responses and systemic resistance in tobacco against tobacco mosaic virus. FEMS Microbiology Letters. 313: 120-126.

Mackie, A.E., Wheatley, R.E. (1999). Effects and incidence of volatile organic compound interactions between soil bacterial and fungal isolates. Soil Biology and Biochemistry. 31: 375-385.

Mahalleh Yoosefi, S.M., Safarzadeh Vishekaei, M.N., Noormohammadi, G., Noorhosseini Niyaki, S.A. (2011). Effect of foliar spraying by methanol on growth of common bean and snap bean in Rasht, North of Iran. Research Journal of Biological Sciences. 2: 47-50.

Malhautier, L., Khammar, N., Bayle, S., Fanlo, J.L. (2005). Biofiltration of volatile organic compounds. Applied Microbiology and Biotechnology. 68: 16-22.

Martinez, A., Obertello, M., Pardo, A., Ocampo, J.A., Godeas, A. (2004). Interactions between *Trichoderma pseudokoningii* strains and the arbuscular mycorrhizal fungi *Glomus mosseae* and *Gigaspora rosea*. Mycorrhiza. 14: 79-84.

Mastouri, F., Bjorkman, T., Harman, G.E. (2010). Seed treatment with *Trichoderma harzianum* alleviates biotic, abiotic, and physiological stresses in germinating seeds and seedlings. Phytopathology. 100: 1213-1221.

Mastouri, F., Bjorkman, T., Harman, G.E. (2012). *Trichoderma harzianum* enhances antioxidant defense of tomato seedlings and resistance to water deficit. Molecular Plant Microbe Interactions. 25: 1264-1271.

Mathivanan, N., Prabavathy, V.R., Vijayanandraj, V.R. (2008). The effect of fungal secondary metabolites on bacterial and fungal pathogens. In: Karlovsky, P. (Ed.) Secondary metabolites in soil ecology. Springer-Verlag. Pgs. 129-140.

Matsui, K., Sugimoto, K., Mano, J., Ozawa, R., Takabayash, J. (2012). Differential metabolisms of green leaf volatiles in injured and intact parts of a wounded leaf meet distinct ecophysiological requirements. PLoS ONE. 7: e36433.

Mayr, D., Margesin, R., Klingsbichel, E., Hartungen, E., Denewein, D., Schinner, F., Märk, T.D. (2003). Rapid detection of meat spoilage by measuring volatile organic compounds by using proton transfer reaction mass spectrometry. Applied and Environmental Microbiology. 69:4697-4705.

Mayrhofer, S., Mikoviny, T., Waldhuber, S., Wagner, A.O., Innerebner, G., Franke-Whittle, I.H., Märk, T.D., Hansel, A., Insam, H. (2006). Microbial community related to volatile organic compound (VOC) emission in household biowaste. Environmental Microbiology. 8: 1960-1974.

McAllister, C.B., Garcia-Romera, I., Godeas, A., Ocampo, J.A. (1994). Interactions between *Trichoderma koningii*, *Fusarium solani*, and *Glomus mosseae*: effects on plant growth, arbuscular mycorrhizas and the saprophyte inoculants. Soil Biology and Biochemistry. 26: 1363-1367.

McNeal, K.S., Herbert, B.E. (2009). Volatile organic metabolites as indicators of soil microbial activity and community composition shifts. Soil Science Society of America Journal. 73: 579-588.

Menzel, T.R., Weldegergis, B.T., David, A., Boland, W., Gols, R., van Loon, J.J.A., Dicke, M. (2014). Synergism in the effect of prior jasmonic acid application on herbivore-induced volatile emission by Lima bean plants: transcription of a monoterpene synthase gene and volatile emission. Journal of Experimental Botany. 65: 4821-4831.

Miao, F.-P., Liang, X.-R., Yin, X.-L., Wang, G., Ji, N.-Y. (2012). Absolute configurations of unique harziane diterpenes from *Trichoderma* species. Organic Letters. 12: 3815-3817.

Minerdi, D., Bossi, S., Gullino, M.L., Garibaldi, A. (2009). Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. Environmental Microbiology. 11: 844-854.

Minerdi, D., Bossi, S., Maffei, M.E., Gullino, M.L., Garibaldi, A. (2011). *Fusarium oxysporum* and its bacterial consortium promote lettuce growth and expansin A5 gene expression through microbial volatile organic compound (MVOC) emission. FEMS Microbiology Ecology. 76: 342-351.

Mitchell, A.M., Strobel, G.A., Moore, E., Robison, R., Sears, J. (2010). Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus. Microbiology. 156: 270-277.

Moeder, Monika. (2014). Gas chromatography-mass spectrometry. In: Dettmer-Wilde, K., Engewald, W. (Ed.) Practical Gas Chromatography. Springer-Verlag Berlin Heidelberg. Pgs. 303-350.

Morales-Barrera, L., Cristiani-Urbina, E. (2008). Hexavlent chromium removal by a *Trichoderma inhamatum* fungal strain isolated from tannery effluent. Water, Air, & Soil Pollution. 187: 327-336.

Morath, S.U., Hung, R., Bennett, J.W. (2012). Fungal volatile organic compounds: a review with emphasis on their biotechnological potential. Fungal Biology Reviews. 26: 73-83.

Mukherjee, P.K., Horwitz, B.A., Singh, U.S., Mukherjee, M., Schmoll, M. (Ed.) (2013). *Trichoderma* Biology and Applications. CAB International. Pgs. 1-9.

Muller, A., Faubert, P., Hagen, M., Zu Castell, W., Polle, A., Schnitzler, J.P., Rosenkranz, M. (2013). Volatile profiles of fungi–chemotyping of species and ecological functions. Fungal Genetics and Biology. 54: 25-33.

Naznin, H.A., Kimura, M., Mizyawa, M., Hyakumachi, M. (2013). Analysis of volatile organic compounds emitted by plant growth-promoting fungus *Phoma* sp. GS8-3 for growth promotion effects on tobacco. Microbes and Environment. 28: 42-49.

Nemcovic, M., Jakubikova, L., Viden, I., Farkas, V. (2008). Induction of conidiation by endogenous volatile compounds in *Trichoderma* spp. FEMS Microbiology Letters. 284: 231-236.

Nonomura, A.M., Benson, A.A. (1992). The path of carbon in photosynthesis: improved crop yields with methanol. Proceedings of the National Academy of Sciences. 89: 9794-9798.

Oikawa, P.Y., Lerdau, M.T. (2013). Catabolism of volatile organic compounds influences plant survival. Trends in Plant Science. 18: 695-703.

Ortiz-Castro, R., Contreras-Cornejo, H.A., Macias-Rodriguez, L., Lopez-Bucio, J. (2009). The role of microbial signals in plant growth and development. Plant Signaling & Behavior. 4: 701-712.

Owen, S., Clark, S., Pompe, M., Semple, K.T. (2007). Biogenic volatile organic compounds as potential carbon sources for microbial communities in soil form the rhizosphere of *Populus tremula*. FEMS Microbiology Letters. 268: 34-39.

Palin, R., Geitmann, A. (2012). The role of pectin in plant morphogenesis. BioSystems. 109: 397-402.

Paknejad, F., Bayat, V., Ardakani, M.R., Vazan, S. (2012). Effect of methanol foliar application on seed yield and it's quality of soybean (*Glycine max* L.) under water deficit conditions. Annals of Biological Research. 3: 2108-2117.

Paul, D., Park, K.S. (2013). Identification of volatiles Produced by *Cladosporium cladosporioides* CL-1, a fungal biocontrol agent that promotes plant growth. Sensors. 13: 13969-13977.

Penuelas, J., Asensio, D., Thol, D., Wenke, K., Rosenkranz, M., Piechulla, B., Schnitzler, J.P. (2014). Biogenic volatile emissions from the soil. Plant, Cell and Environment. 37: 1866-1891.

Pinches, S.E., Apps, P. (2007). Production in food of 1,3-pentadiene and styrene by *Trichoderma* species. International Journal of Food Microbiology. 116: 182-185.

Polizzi, V., Adamns, A., Malysheva, S.V., De Saeger, S., Van Peteghem, C., Moretti, A., Picco, A.M., De Kimpe, N. (2012). Identification of volatile markers for indoor fungal

growth and chemotaxonomic classification of *Aspergillus* species. Fungal Biology. 116: 941-953.

Prost, I., Dhondt, S., Rothe, G., Vicente, J., Rodriguez, M.J., Kift, N., Carbonne, F., Griffifths, G., Esquerré-Tugayé, M.T., Rosahl, S., Castresana, C., Hamberg, M., Fournier, J. (2005). Evaluation of the antimicrobial activities of plant oxylipins supports their involvement in defense against pathogens. Plant Physiology. 139: 1902-1913.

Ramadan, T., Omran, Y.A.M.M. (2005). The effect of foliar application of methanol on productivity and fruit quality of grapevine cv. Flame Seedless. Vitis. 44: 11-16.

Ramirez, I., Dorta, F., Espinoza, V., Jimenez, E., Mercado, A., Pena-Cortes, H. (2006). Effects of foliar and root applications of methanol on the growth of *Arabidopsis*, tobacco, and tomato plants. Journal of Plant Growth Regulation. 25: 30-44.

Ramirez, K.S., Lauber, C.L., Fierer, N. (2009). Microbial consumption and production of volatile organic compounds at the soil-litter interface. Biogeochemistry. 99: 97-107.

Rigamonte, T.A., Pylro, V.S., Duarte, G.F. (2010). The role of mycorrhization helper bacteria in the establishment and action of ectomcyorrhizae associations. Brazilian Journal of Microbiology. 41: 832-840.

Ruiz, J., Bilbao, R., Murillo, M.B. (1998). Adsorption of different VOC onto soil minerals from the gas phase: influence of mineral, type of VOC, and air humidity. Environmental Science & Technology. 32: 1079-1084.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wie, H.-X., Pare, P.W., Kloepper, J.W. (2003). Bacterial volatiles promote growth of *Arabidopsis*. Proceedings of the National Academy of Sciences. 100: 4927–4932.

Santino, A., Taurino, M., De Domenico, S., Bonsegna, S., Poltronieri, P., Pastor, V., Flors, V. (2013). Jasmonate signaling in plant development and defense response to multiple abiotic stresses. Plant Cell Reports. 32:1085-1098.

Saxena, S., Meshram, V., Kapoor, N. (2014). *Muscodor tigerii* sp. nov.-volatile antibiotic producing endophytic fungus from the Northeastern Himalayas. Annals of Microbiology. doi: 10.1007/s13213-014-0834-y.

Schade, G.W., Custer, T.G. (2004). VOC emissions from agricultural soil in northern Germany during the 2003 European heat wave. Atmospheric Environment. 38: 6105-6114.

Schade, G.W., Goldstein, A.H. (2001). Fluxes of oxygenated volatile organic compounds from a ponderosa pine plantation. Journal of Geophysical Research-Atmospheres. 106: 3111-3123.

Schalchli, H., Hormazabal, E., Becerra, J., Birkett, M., Alvear, M., Vidal, J., Quiroz, A. (2011). Antifungal activity of volatile metabolites emitted by mycelial cultures of saprophytic fungi. Chemistry and Ecology. 27: 503-513.

Schluter, M., Gentz, T. (2008). Application of membrane inlet mass spectrometry for online and in situ analysis of methane in aquatic environments. Journal of the American Society for Mass Spectrometry. 19: 1395-1402.

Schöller, C., Molin, S., Wilkins, S. (1997). Volatile metabolites from some gram-negative bacteria. Chemosphere. 35: 1487-1495.

Schuster, A., Schmoll, M. (2010). Biology and biotechnology of *Trichoderma*. Applied Microbiology and Biotechnology. 87: 787-799.

Seewald, M.S.A., Bonfanti, M., Singer, W., Knapp, B.A., Hansel, A., Franke-Whittle, I., Insam, H. (2010). Substrate induced VOC emissions from compost amended soils under aerobic and anaerobic incubation. Biology and Fertility of Soils. 46: 371-382.

Serrano, A., Gallego, M. (2006). Sorption study of 25 volatile organic compounds in several Mediterranean soils using headspace–gas chromatography–mass spectrometry. Journal of Chromatography. 1118: 261-270.

Sheppard, S.K., Lloyd, D. (2002). Effects of soil amendment on gas depth profiles in soil monoliths using direct mass spectrometric measurement. Bioresource Technology. 84: 39-47.

Shoresh, M., Harman, G.E., Mastouri, F. (2010). Induced systemic resistance and plant responses to fungal biocontrol agents. Annual Review of Phytopathology. 48: 21-43.

Siddiquee, S., Cheong, B.E., Taslima, K., Kausar, H., Hasan, M. (2012). Separation and identification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC-MS using three different capillary columns. Journal of Chromatographic Science. 50: 358-367.

Singh, S.K., Strobel, G.A., Knighton, B., Geary, B., Sears, J., Ezra, D. (2011). An endophytic *Phomopsis* sp. possessing bioactivity and fuel potential with its volatile organic compounds. Microbial Ecology. 61: 729-739.

Splivallo, R., Novero, M., Bertea, C.M., Bossi, S., Bonfante, P. (2007). Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist. 175: 417-424.

Steyaert, J.M., Weld, R.J., Mendoza-Mendoza, A., Stewart, A. (2010). Reproduction without sex: conidiation in the filamentous fungus *Trichoderma*. Microbiology. 156: 2887-2900.

Stinson, M., Ezra, D., Hess, W.M., Sears, J., Strobel, G. (2003). An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. Plant Science. 165: 913-922.

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., Schuhmacher, R. (2010). Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. Journal of Microbiological Methods. 81: 187-193.

Stotzky, G., Schenck, S. (1976). Volatile organic compounds and microorganisms. Critical Reviews in Microbiology. 4: 333-382.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C. (2001). Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Mirobiology. 147: 2943-2950.

Szabo, M., Csepregi, K., Galber, M., Viranyi, F., Fekete, C. (2012). Control plant-parasitic nematodes with *Trichoderma* species and nematode-trapping fungi: the role of chi18-5 and chi18-12 genes in nematode egg-parasitism. Biological Control. 63: 121-128.

Tait, E., Perry, J.D., Stanforth, S.P., Dean, J.R. (2014). Identification of volatile organic compounds produced by bacteria using HS-SPME-GC-MS. Journal of Chromatographic Science. 52: 363-373.

Tholl, D., Boland, W., Hansel, A., Loreto, F., Röse, U.S.R., Schnitzler, J.-P. (2006). Practical approaches to plant volatile analysis. The Plant Journal. 45: 540-560.

Tripathi, P., Singh, P.C., Mishra, A., Chauhan, P.C., Dwivedi, S., Bais, R.T., Tripathi, R.D. (2013). *Trichoderma*: a potential bioremediatior for environmental clean up. Clean Technologies and Environmental Policy. 15: 541-550.

Tsahouridou, P.C., Thanassoulopoulos, C.C. (2002). Proliferation of *Trichoderma koningii* in the tomato rhizosphere and the suppression of damping-off by *Sclerotium rolfsii*. Soil Biology & Biochemistry. 34: 767-776.

Ul-Hassan, S.R., Strobel, G.A., Booth, E., Knighton, B., Floerchinger, C., Sears, J. (2012). Modulation of volatile organic compound formation in the Mycodiesel-producing endophyte *Hypoxylon* sp. CI-4. Microbiology. 158: 465-473.

van Roon, A., Parsons, J.R., Te Kloeze, A.-M., Govers, H.A.J. (2005a). Fate and transport of monoterpenes through soils. Part I: prediction of temperature dependent soil fate model input-parameters. Chemosphere. 61: 599-609.

van Roon, A., Parsons, J.R., Krap, L., Govers, H.A.J. (2005b). Fate and transport of monoterpenes through soils. Part II: calculation of the effect of soil temperature, water saturation and organic carbon content. Chemosphere. 61: 129-138.

Vargas, W.A., Mandawe, J.C., Kenerley, C.M. (2009). Plant-derived sucrose is a key element in the symbiotic association between *Trichoderma virens* and maize plants. Plant Physiology. 151: 792-808.

Vespermann, A., Kai, M., Piechulla, B. (2007). Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Applied Environmental Microbiology. 73: 5639-5641.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L., Lorito, M. (2008). *Trichoderma*-plant pathogens interactions. Soil Biology and Biochemistry. 40: 1-10.

Wan, M., Li, G., Zhang, J., Jiang, D., Huang, H.C. (2008). Effect of volatile substances of *Streptomyces platensis* F-1 on control of plant fungal diseases. Biological Control. 46: 552-559.

War, A.R., Sharma, H.C., Paulraj, M.G., War, M.Y., Ignacimuthu, S. (2011). Herbivore induced plant volatiles: their role in plant defense for pest management. Plant Signaling & Behavior. 6: 1973-1978.

Wheatley, R.E. (2002). The consequences of volatile organic compound mediated bacterial and fungal interactions. Antonie van Leeuwenhoek. 81: 357-364.

Wheatley, R., Hackett, C., Bruce, A., Kundzewicz, A. (1997). Effect of substrate composition on production of volatile organic compounds from *Trichoderma* spp. inhibitory to wood decay fungi. International Biodeterioration & Biodegradation. 39: 199-205.

Whipps, J.M., Lumsden, R.D. (Ed.) (1989). Biotechnology of fungi for improving plant growth. Cambridge Univeristy Press.

Wilkins, K., Larsen, K., Simkus, M. (2000). Volatile metabolites from mold growth on building materials and synthetic media. Chemosphere. 41: 437-446.

Winberry Jr., W.T., Jungclaus, G. (1999). Compendium method TO-13A – determination of polycyclic aromatic hydrocarbons (PAHs) in ambient air using gas chromatography/ mass spectrometry (GC/MS). U.S. Environmental Protection Agency, Cincinnati.

Wong, P.S.H., Cooks, R.G., Cisper, M.E., Hemberger, P.H. (1995). Online, in-situ analysis with membrane introduction MS. Environmental Science & Technology. 29: 215–218.

Wrigley, D.M. (2004). Inhibition of *Clostridium perfringens* sporulation by *Bacteroides fragilis* and short-chain fatty acids. Anaerobe. 10: 295-300.

Xiao-Yan, S., Qing-Tao, S., Shu-Tao, X., Xiu-Lan, C., Cai-Yun, S., Yu-Zhong, Z. (2006). Broad-spectrum antimicrobial activity and high stability of Trichokonins from *Trichoderma koningii* SMF2 against plant pathogens. FEMS Microbiology Letter. 260: 119-125.

Yang, Z., Yu, Z., Lei, L., Xia, Z., Shao, L., Zhang, K., Li, G. (2012). Nematicidal effect of volatiles produced by *Trichoderma* sp. Journal of Asia-Pacific Entomology. 15: 647-650.

Zhang, H., Kim, M.-S., Krishnamachari, V., Payton, P., Sun, Y., Grimson, M., Farag, M.A., Ryu, C.M., Allen, R., Melo, I.S., Pare, P.W. (2007). Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. Planta. 226: 839-851.

Zhao, L.J., Yang, X.N., Li, X.Y., Mu, W., Liu, F. (2011). Antifungal, insecticidal and herbicidal properties of volatile components from *Paenibacillus polymyxa* Strain BMP-11. Agricultural Sciences in China. 10: 728-736.

Zeilinger, S., Schumacher, R. (2013). Volatile organic metabolites of *Trichoderma* spp.: biosynthesis, biology and analytics. In: Mukherjee, P.K., Horwitz, B.A., Singh, U.S., Mukherjee, M., Schmoll, M. (Ed.) *Trichoderma:* Biology and Applications. CAB International. Pgs. 110-127.

CHAPTER 2.

Age Matters: The Effects of Volatile Organic Compounds Emitted by *Trichoderma atroviride* on Plant Growth

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2.1 Introduction

Numerous parameters influence the qualitative and quantitative aspects of volatile production. In soil habitats, the profile of VOCs varies with inorganic soil constituents, micronutrient content, microbial community composition, biomass, and environmental conditions such as temperature, humidity and pH (McNeal and Herbert, 2009; Insam and Seewald, 2010). In addition, the concentration and duration of volatile exposure play a critical role when measuring plant responses. Further complicating the picture, different laboratories have used somewhat different experimental designs. All of these factors make it difficult to interpret experimental results between laboratories. For example, one of the most commonly reported volatiles emitted by fungi, 1-octen-3-ol (mushroom alcohol), is a dominant component of the odor of cultivated mushrooms and moldy indoor air, and is responsible for a wide range of toxic and beneficial effects in cell culture, human subjects, microorganisms, and plants. Several studies have demonstrated the phytoxicity of this compound in *Arabidopsis* (Combet et al., 2006; Splivallo et al., 2007; Hung et al., 2014; Lee et al., 2014). In contrast, exposure to 1-octen-3-ol had enhanced plant resistance to the pathogen *Botrytis cineregand* (Kishimoto et al., 2007).

Our laboratory has focused on studying the effects of filamentous fungal volatiles on plants using *Arabidopsis* as a model. Previously, we demonstrated that *Arabidopsis thaliana* grown in the presence of *Trichoderma viride* VOCs exhibited increased biomass and chlorophyll concentration (Hung et al., 2013). In the course of our literature reviews, we have learned that there is considerable inter-laboratory variability in the design of the exposure systems to study microbial volatiles leading to contrasting results (Fiddaman and Rossall, 1993; Stinson et al., 2003; Ryu et al., 2004; Atmosukarto et al., 2005; Splivallo et al., 2007; Blom et al., 2011). Using a simple and reproducible method for

conducting volatile exposure studies on *Arabidopsis* with filamentous fungi, we investigated the effect of VOCs emitted by *Trichoderma atroviride.* We present evidence that both the age of the fungal culture and the exposed plant material is very important to the growth modulation effects. Depending on the conditions, VOCs from the same *Trichoderma* isolate can either stimulate plant growth or induce toxicity.

2.2 Materials and methods

Trichoderma and Arabidopsis Growth Conditions

Trichoderma atroviride (GJS 01-209) was obtained from Amy Rossman at USDA-ARS, Beltsville, MD. The fungus was grown in 35x10 mm petri dish on 4 ml of malt extract agar (MEA) and incubated for five days at 27 $\pm$1 ˚C in high humidity prior to the start of the volatile exposure experiments. *Arabidopsis thaliana* seeds (ecotype Columbia-7) were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). The plant seeds were surface-sterilized as described previously using ethanol and bleach solution (Hung et al., 2014). Five surface sterilized seeds were sown onto a 100 x 15 mm partitioned petri dish (split- or I-plate) containing Murashige and Skoog medium with vitamins, 3% sucrose, 0.03% phytagel (pH 5.7) (Phytotechnology Laboratories, KS). Seeds were stratified at 4˚C for three days prior to volatile exposure.

Plant Exposure to Trichoderma VOCs

For the double plate-within-a-plate system, plate containing *Trichoderma* was placed into the partitioned Petri dish containing the stratified plant seeds, or one week old plant seedlings. Plant and fungi were grown together in a growth chamber at 23 $\pm$1 ˚C with a 16-hour photoperiod for 14 days. For controls, the same plate-within-a-plate system was used with sterile media. At the end of the exposure period, the plants were

removed from the exposure conditions; the shoots were separated from the roots, and weighed before the total chlorophyll concentration was determined. Three replicates were used per treatment conditions and the experiments were repeated three times. Quantitative results were expressed as standard error of the mean and analyzed using Excel software (Microsoft, Redmond, WA) and SigmaPlot (SPSS Science Inc., IL). Student's t-test and one-way analysis of variance (ANOVA) between groups were performed for all quantitative data.

Plant Chlorophyll Measurements

Total chlorophyll concentration of plants exposed to *Trichoderma* VOCs was determined using the method described previously (Hung et al., 2013). Fresh shoot tissue was submerged overnight in 1 ml of 80% acetone in the dark at 4˚C. The total chlorophyll concentration (chlorophyll a and b) was calculated from the equation, ((8.02)(A663) + (20.2)(A645))V/1000W, where V is volume and W is plant fresh weight. The chlorophyll data were expressed in relation to the fresh weight of the plant shoot.

VOC Analysis by Headspace GC-MS

VOC capture and analysis were conducted as described previously (Hung et al., 2013). Using a purge and trap method, *Trichoderma* was grown on MEA for 5 days or 14 days at 27 ˚C. Controls consisted of headspace samples taken over uninoculated fungal media. The headspace of the container was purged at 100 ml per min for four hours. The VOCs were adsorbed on 6 cm Tenax columns (Scientific Instrument Services, Ringoes, NJ), 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 (SigmaAldrich Corp., St. Louis, MO) column: 0.32 mm diameter, 1 mm film

thickness. The compounds 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: 10:1 split, helium carrier at 20 psi, oven temperature from -20 ˚C to 280 ˚C at 10 ˚C per min. The MS conditions were: positive ion mode, electron impact spectra at 70 eV. The MS of the peaks were determined by their scatter pattern. Internal standards were used to normalize the peak areas.

2.3 Results and discussions

Studying volatile-mediated interaction between plants and microorganisms is extremely challenging because of the distinct chemical properties of VOCs; and the fact that these compounds occur in dynamically changing complex mixtures that vary with producing species, as well as age of culture, growth media and other environmental parameters (Hutchinson, 1973; Korpi et al., 2009; Herrmann, 2010; Bitas et al., 2013). Furthermore, it is difficult to compare data produced in different laboratories because of variations in experimental design in plant exposure. We found that the standard split-plate (I-plate) method, commonly used in bacteria-plant volatile exposure studies (Ryu et al., 2004; Gutierrez-Luna et al., 2010; Blom et al., 2011), was associated with frequent contamination issues where the aggressive mycelial nature of *Trichoderma* colonized the plants directly, preventing further study.

Using our modified plate-within-a-plate method, we obtained more uniform fungal growth while eliminating overgrowth of fungi and contamination of plants. Importantly, we found that the plant growth response to fungal VOCs was dependent on the age of fungal culture emitting VOCs and the growth stage of plants being exposed to these volatiles. As seen on Figure 1, *Arabidopsis* seeds exposed to five-day-old *T.*

atroviride VOCs for 14 days exhibited reduction in plant size, formation of necrotic lesions, and loss of chlorophyll. Since fungal samples can be easily exchanged in this exposure system, we were able to study the timeline of the VOC production from the fungi on one hand and plant response on the other (Fig. 2a, 2b). At the end of 14 days, the average total chlorophyll concentration of control plants was 0.31 $\pm$0.04 mg per gram of fresh shoot tissue (mg/g). *Arabidopsis* seeds exposed to freshly inoculated fungal culture (zero-day-old) were similar to control plants; neither growth (shoot fresh weight) nor chlorophyll concentration was affected during the two week exposure. However, when germinating seeds were exposed to a five-day-old culture, plants exhibited yellowed and bleached leaves with concomitant reduction in chlorophyll concentration at 0.14 $\pm$0.03 mg/g of chlorophyll. The effect was no more pronounced when the culture of five-day-old fungal culture was replaced with another plate of actively growing fungal culture (five-day-old) at day seven (0.20 $\pm$0.04 mg/g). When seven-day-old seedlings were exposed to a five-day-old culture of the fungus, plants exhibited significant increase in growth with an average of 0.49 $\pm$ 0.6 mg/g chlorophyll production. Fresh shoot weights of *Arabidopsis* exposed to *Trichoderma* VOCs correlated with the total chlorophyll concentration data and the data collected were significantly different from controls.

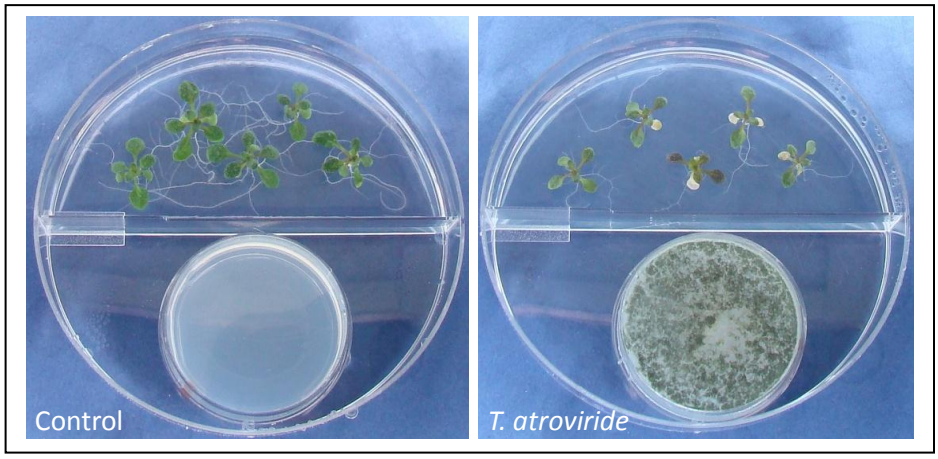


Figure 1. General growth inhibition of *Arabidopsis thaliana* exposed to five-day-old *Trichoderma atroviride* VOCs for fourteen days.

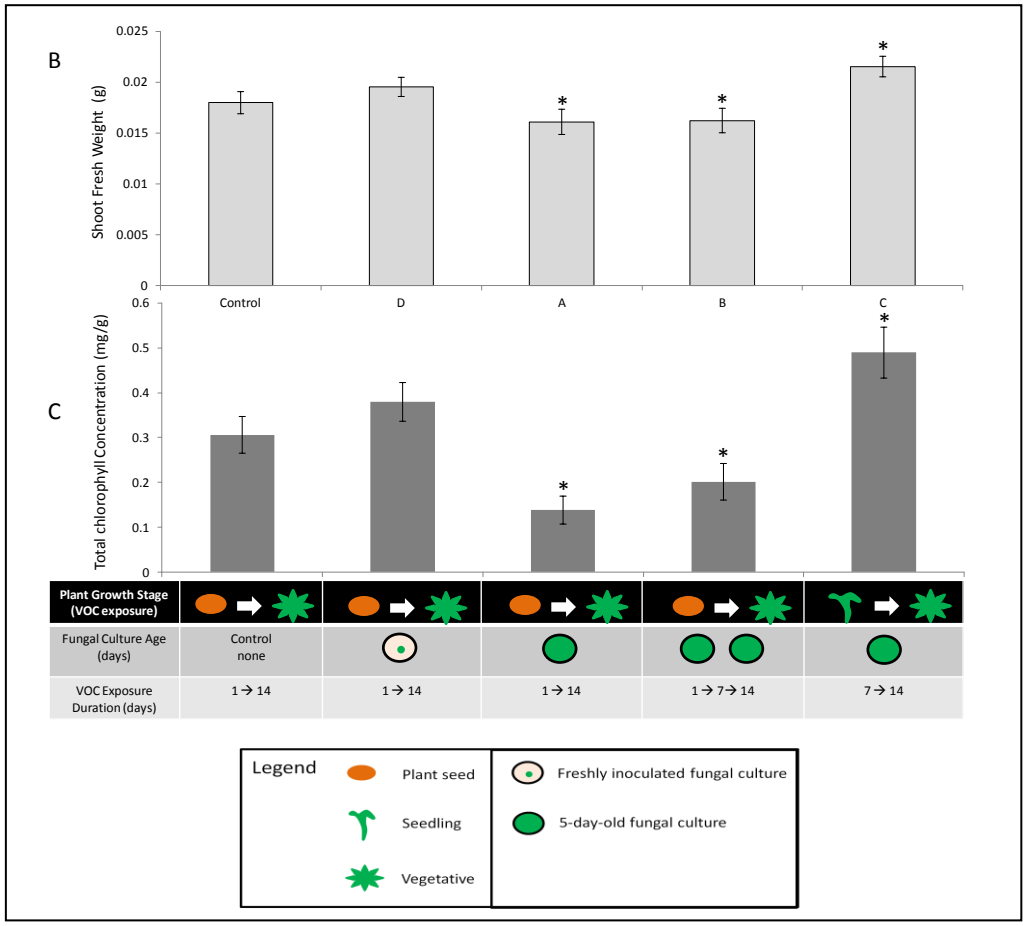


Figure 2. *Arabidopsis thaliana* exposed to *Trichoderma atroviride* VOCs. Plant response to *T. atroviride* VOCs at different fungal culture age. (A) Average shoot fresh weight of *A. thaliana*. (B) Total chlorophyll concentration of plants exposed to *Trichoderma* VOCs. * indicates significance (ANOVA, $P = 0.001$).

During the course of our volatile exposure experiments fungal colonies mature; therefore, we compared the fungal volatile emission of five-day-old and fourteen-day-old *T. atroviride* using GC-MS (Table 1). We identified a total of 56 unique compounds that were produced by *Trichoderma*. Most of the detected VOCs were aromatics, alcohols, ketones, aldehydes or alkenes. As the fungi matured, some compounds were lost while new compounds were produced. The accumulation of these differentially-produced VOCs may account for the differential effects observed on plant growth. Compounds, 6-pentyl-pyran-2-one (6PP) and acetoin, identified in this study have been shown to induce plant growth (Ryu et al., 2003; Vinale et al., 2008). In our study, 6PP was produced at the highest concentration by 5- and 14-day-old cultures (at 3069.25 ng/trap and 3270.74 ng/trap respectively) and very small concentration of acetoin was produced in both cultures. Presence of these compounds does not explain the severe growth inhibition observed in the plants when exposed to 5-day-old fungal cultures for 14 days. It is also interesting that *T. viride* volatile profile from our previous study was different from this study and 6PP was not present (Hung et al., 2013). For these reasons, determining this fungal isolate to induce volatile-mediated growth promotion is difficult.

In this work, we have presented an improved experimental design for studying plant exposure to filamentous fungal VOCs and have shown that plant growth response to fungal VOCs is dependent on age of both the plant and the fungal cultures. Some of the conflicting reports on microbial volatile-mediated growth promotion and/or inhibition may be attributed to differences in exposure parameters. Careful consideration needs to be made when assessing volatiles for growth promotion.

MS Scan #	Peak Assignment	5-day-old	14-day-old
303	ethanol	x	x
318	acetone	x	x
350	isopropanol		x
384	2-methylpropanal	x	x
395	acrylonitrile		x
434	butanal	x	x
442	ethyl acetate	x	x
450	2-butanone	x	x
505	3-methylbutanal	x	x
512	2-methylbutanal	x	x
525	2-pentanone		x
546	1-butanol		x
575	propyl acetate	x	
612	pentyl alcohol	x	x
627	acetoin	x	x
647	butyl acetate		x
662	hexanal	x	x
674	2-methoxymethylfuran	x	x
684	*2-methoxymethylfuran + pyrrole*		*x*
702	pentyl acetate	x	
737	styrene		x
747	furfural		x
752	2-heptanone	x	x
757	heptanal		x
758	2-heptanol	x	
775	furfuryl alcohol		x
812	2-acetylfuran		x
832	2-ethylhexanal	x	x
837	2-octanone	x	x
846	octanal	x	x
855	benzaldehyde	x	x
874	2-ethylhexyl alcohol		x
902	2-nonanone	x	x
917	nonanal	x	x
950	acetophenone	x	x
959	nonyl alcohol		x
1003	2-decanone	x	x
1009	decanal	x	x
1036	methyl salicylate		x
1074	2-undecanone	x	x
1082	undecanal	x	
1083	1,2,3,4-tetrahydro-5-methyl-naphthalene		x
1112	2-phenoxyethanol		x
1132	alpha-farnesene	x	x
1154	chlorinated butadiene oligomer	x	x
1197	sesquiterpene		x
1199	alpha-bergamtoene	x	x
1220	beta-farnesene	x	x
1228	isocaryophyllene		x
1258	n-heptadecane		x
1266	6-pentyl-pyran-2-one	x	x
1317	6-pentyl-pyran-2-one (unsaturated)	x	x
1320	204 mw sesquiterpene		x
1357	methyl dihydrojasmonate		x
1379	isopropyl laurate		x
1408	octanal-2-(phenylmethylene)		x

Table 1. Headspace volatiles collected from five-day-old and fourteen-day-old *Trichoderma atroviride* at 100 ml/min, purge rate, 4 hours, 1.0 μg Int. Std. by P&T-TD-GC-MS. x denotes presence of compound.

REFERENCES

Atmosukarto, I., Castillo, U., Hess, W.M., Sears, J., Strobel, G. (2005). Isolation and characterization of *Muscodor albus* I-41.3s, a volatile antibiotic producing fungus. Plant Science. 169: 854-861.

Bitas, V., Kim, H.S., Bennett, J.W., Kang, S. (2013). Sniffing on microbes: diverse roles of microbial volatile organic compounds in plant health. Molecular Plant-Microbe Interactions Journal. 26: 835-43.

Blom, D., Fabbri, C., Eberl, L., Weisskopf, L. (2011). Volatile-mediated killing of *Arabidopsis thaliana* by bacteria is mainly due to hydrogen cyanide. Applied and Environmental Microbiology. 77: 1000-1008.

Combet, E., Henderson, J., Eastwood, D.C., Burton, K.S. (2006). Eight-carbon volatiles in fungi: properties, analysis, and biosynthesis. Mycoscience. 47: 317-326.

Fiddaman, P.J., Rossall, S. (1993). The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology. 74: 119-126.

Gutiérrez-Luna, F.M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., de la Cruz, H.R., Macías-Rodríguez, L. (2010). Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. Symbiosis. 51: 75-83.

Herrmann, A. (Ed.) (2010). The Chemistry and Biology of Volatiles. John Wiley & Sons.

Hung, R., Lee, S., Bennett, J.W. (2013). *Arabidopsis thaliana* as a model system for testing the effects of *Trichoderma* volatile organic compounds. Fungal Ecology. 6: 19-26.

Hung, R., Lee, S., Bennett, J.W. (2014). The effects of low concentrations of the enantiomers of mushroom alcohol (1-octen-3-ol) on *Arabidopsis thaliana*. Mycology: An International Journal on Fungal Biology. 5: 73-80.

Hung, R., Lee, S., Rodriguez-Saona, C., Bennett, J.W. (2014). Common gas phase molecules from fungi affect seed germination and plant health in *Arabidopsis thaliana*. AMB Express. 4: 53. doi:10.1186/s13568-014-0053-8.

Hutchinson, S.A. (1973). Biological activities of volatile fungal metabolites. Annual Review of Phytopathology. 11: 233-246.

Insam, H., Seewald, S.A. (2010). Volatile organic compounds (VOCs) in soils. Biology and Fertility of Soils. 46: 199-213.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J. (2007). Volatile 1-octen-3-ol induces a defensive response in *Arabidopsis thaliana*. Journal of Plant Pathology. 73: 35-37.

Korpi, A., Jarnberg, J., Pasanen, A.L. (2009). Microbial volatile organic compounds. Critical Reviews in Toxicology. 39: 139-193.

Lee, S., Hung, R., Schink, A., Mauro, J., Bennett, J.W. (2014). *Arabidopsis thaliana* for testing the phytotoxicity of volatile organic compounds. Plant Growth Regulation. 74: 177-186.

McNeal, L., Herbert, B. (2009). Volatile organic metabolites as indicators of soil microbial activity and community composition shifts. Soil Science Society of America. 73: 579-588.

Stinson, M., Ezra, D., Hess, W.M., Sears, J., Strobel, G. (2003). An endophytic *Gliocladium* sp. of *Eucryphia cordifolia* producing selective volatile antimicrobial compounds. Plant Science. 165: 913-922.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Wei, H.X., Paré, P.W., Kloepper, J.W. (2003). Bacterial volatiles promote growth in *Arabidopsis*. Proceedings of the National Academy of Sciences. 100: 4927-4932.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Kloepper, J.W., Paré, P.W. (2004). Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiology. 134: 1017-1026.

Splivallo, R., Novero, M., Bertea, C.M., Bossi, S., Bonfante, P. (2007). Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist. 175: 417-424.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Barbetti, M.J., Li, H., Woo, S.L., Lorito, M. (2008). A novel role for *Trichoderma* secondary metabolites in the interactions with plants. Physiological and Molecular Plant Pathology. 72: 80-86.

CHAPTER 3.

Arabidopsis thaliana for Testing the Phytotoxicity of Volatile Organic Compounds

Lee, S., Hung, R., Schink, A., Mauro, J., Bennett, J.W. (2014). Phytotoxicity of volatile organic compounds. Plant Growth Regulation. 74: 177-186.

3.1 Introduction

Volatile organic compounds (VOCs) are low molecular weight carbon compounds that easily become gases at room temperature, readily migrate in the environment, and often have distinctive odorant properties (Hermann, 2010). VOCs are ubiquitous in the atmosphere and have been studied extensively as fragrance and flavor compounds (Berger, 2007). Many environmental pollutants are volatiles; industrial VOCs that have been well studied for their toxicity include benzene, formaldehyde, and toluene (Nikolaou et al., 2002; Zogorski et al., 2006; Juang et al., 2010). A myriad of other anthropogenic VOCs are emitted as mixtures from adhesives, deodorants, fuels, pesticides, refrigerants, industrial solvents and the like are theorized to have adverse effects on atmospheric conditions and human health (Steiner et al., 2006; Ebi and McGregor, 2008), especially in indoor environments (Baechler et al., 1991; Straus, 2004). Moreover, plants and microbes also produce large numbers of VOCs (Wilkins et al., 2000; Hermann, 2010; Korpi et al., 2009). In particular, the presence of molds in damp indoor environments have received attention as possible contributors to adverse human health effects sometimes called “sick building syndrome” (Li and Yang, 2004; WHO, 2009) and it has been hypothesized that fungal VOCs are responsible for at least some of these negative health effects (Mølhave, 2009; Takigawa et al., 2009).

The possible toxic effects of biogenic VOCs on plant growth and development have received limited research attention; most toxicological studies have been conducted using animal based bioassays. For example, one of the most abundant fungal VOCs, 1-octen-3-ol (“mushroom alcohol”), a dominant component of the odor of cultivated mushrooms and moldy-indoor air, has demonstrable toxic effects in cell culture and on

human subjects (Walinder et al., 2005; Korpi et al., 2009). Geosmin, a bicyclic alcohol, accounts for the characteristic muddy and musty odors associated with soil. It functions in signaling, displays antibiotic activities, inhibits seed germination in Brassicaceae and serves as an avoidance cue for *Drosophila* (Ogura et al., 2000; Watson, 2003; Stensmyr et al., 2012).

Seed germination assays, especially using lettuce, *Lactara sativa*, and cucumber, *Cucumis sativus*, have been developed as rapid and low cost phytotoxicity tests that can be used to monitor the effects of both single compounds and complex mixtures (EPA, 1994; Banks and Schultz, 1995; Wang et al., 2001). Most of these toxicological surveys have studied compounds in aqueous solution related to issues of soil and water purity. The possible phytotoxicity of gas-phase molecules has received far less attention. We hypothesized that both biogenic and anthropogenic VOCs would have detectable negative effects on seed germination and vegetative plant growth, and that certain air freshener products used to mask unpleasant odors might also be phytotoxic. The goal of our study was to develop a simple *Arabidopsis thaliana* bioassay for testing the phytotoxic effects of vapor-phase compounds. Our specific objective was to compare the phytotoxicity of major odorant VOCs emitted by molds, with some common air freshener products used to mask their odors. As positive controls we included three organic solvents commonly found in these air freshener products: ethanol, formaldehyde and isopropyl alcohol. Commonly used household fragrance products are regarded as safe and due to proprietary reasons, exact chemical components are unknown. We tested at concentrations similar to those found in previous studies of fragranced products. In our study, both *A. thaliana* seeds and two-week-old vegetative plants were exposed to a shared atmosphere of 1 ppm

of fourteen different VOC treatments for 72 hours. Phytotoxicity was assayed by the rate of germination and seedling formation; morphological changes in vegetative plant growth; and chlorophyll concentration of shoots and leaves.

3.2 Materials 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 30 minutes. 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% phytagel (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 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). Chemical standards of seven VOC commonly produced by fungi were used in this experiment: geosmin, 1-octanol, 2-octanol, 3-octanol, 3-octanone, 1-octen-3-ol, and 1-octen-3-one. As positive controls we included three organic solvents: ethanol, formaldehyde and isopropyl alcohol. Four commercially available fragranced products

containing mixtures of VOC with various delivery mechanisms were used. For proprietary reasons, the exact composition of the fragrance products is not available; however, the odor description and hazards identification are given in Table 1B. Previously, we determined that there were no significant differences when plants were expose to single compounds at concentration calculated in mass to volume (mg/m^3) versus volume to volume (µl/l). For this study, the 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. An appropriate aliquot of each individual VOC or VOC mixture in liquid form was added, the jar was sealed with a translucent polypropylene screw cap, and the compound volatilized. The seeds were exposed to VOCs for 72 hours in a growth chamber at 23$\pm$1 ˚C with a 16-hour 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. 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) (Fig. 2). Seeds scored as “no germination” included seeds with a ruptured

testa (seed coat) but without the presence of the radicle. Three replicates (90 seeds total) were used per treatment and the experiments were repeated four times.

Vegetative Exposure to VOCs

Prior to exposure, *A. thaliana* plants were grown in individual test tubes for 14 days in a growth chamber at 23±1 ˚C with a 16-hour photoperiod. Then four 14-day-old plants in individual test tubes were placed together into a glass tissue culture jar with one liter of free volume. One ppm of each VOC was added and the jar was sealed with translucent screw caps. The plants were exposed to each of the VOCs for the duration of 72 hours in a growth chamber at 23±1 ˚C with a 16-hour 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 observed for leaf size, leaf color, and other morphological features. Then 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 weight was obtained from the aerial portion of the plant. After weighing, the stems and leaves of each plant were submerged separately in 1 ml of 80% acetone and extracted overnight 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)(A663) + (20.2)(A645))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.

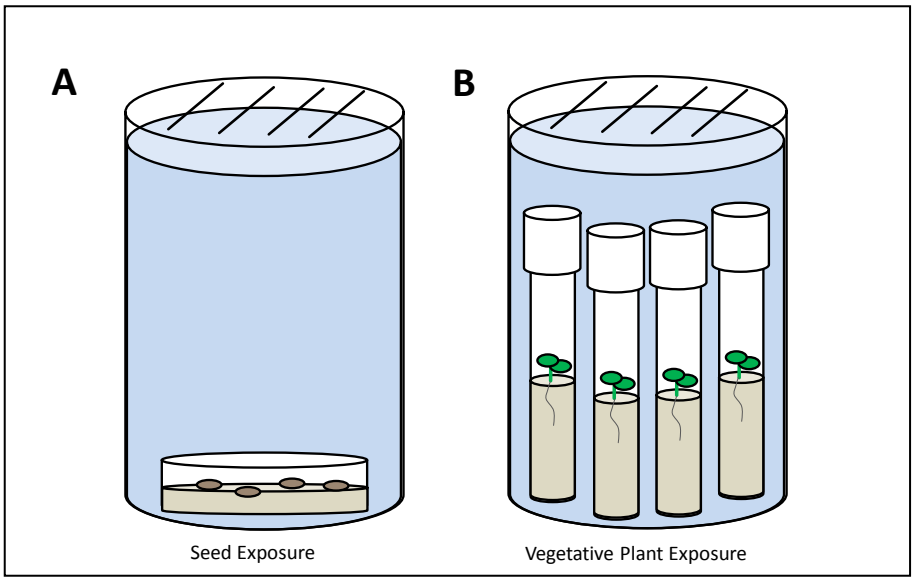


Figure 1. VOC exposure setup. (A) Germination and seeding formation. (B) Vegetative plant exposure.

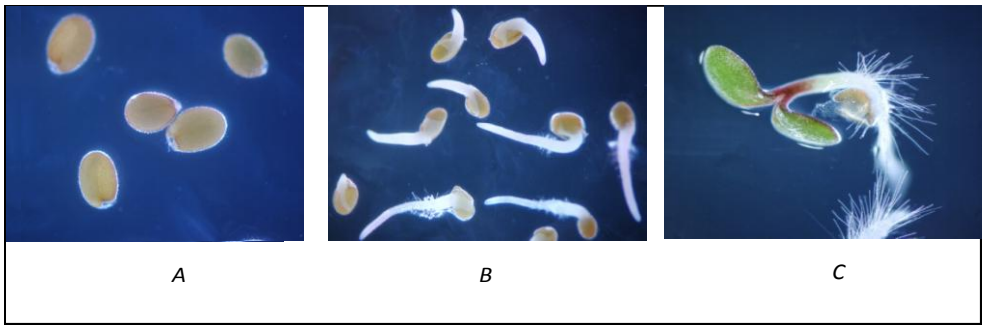
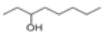
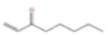


Figure 2. *Arabidopsis thaliana* seeds. (A) No germination, (B) Germination, (C) Progression to seedling.

(A)

Name (purity)	CAS	Molecular formula	Molecular weight	Structure
(a)				
Ethanol (97 %)	64-17-5	C_2H_60	46.1	
Formaldehyde (37 %)	50-00-0	CH_20	30.0	
Isopropyl alcohol (99 %)	67-76-0	C_3H_8O	60.1	
Geosmin (98 %}	19700-21-1	$C_{12}H_{22}0$	182.3	
1-Octanol (99 %)	111-87-5	$C_gH_{18}0$	130.23	
2-Octanol (97 %)	123-96-6	$C_8H_{18}0$	130.23	
3-Octanol (99 %)	20296-29-1	$C_8H_{18}0$	130.23	
3-Octanone (98 %)	106-68-3	$C_8H_{16}0$	128.21	
l-Octen-3-ol (98 %)	3391-86-4	$C_8H_{16}0$	128.21	
l-Octen-3-one (98 %)	4312-99-6	$C_8H_{14}0$	126.20	

(B)

Name	Fragrance delivery mechanisms	Odor description	MSDS hazards identification
(b)			
Air freshener spray	Aerosol canister with compressed nitrogen propellant	Floral with musk	Irritation–inhaled or direct skin contact Proprietary fragrance mixtures Ethanol
Stick fragrance oil	Fragrance oil with wooden stick to aid fragrance dispersal	Rose	Irritation–inhaled or direct skin contact Proprietary fragrance mixtures
Scented body mist/spray	Aerosol spray canister, apply directly onto body or clothing	Lemon, citrus	Not available Proprietary fragrance mixtures
Scented oil	Equipped with micropore membrane to allow slow release of scented oils over time	Tropical fruit mix	Prolonged exposure may cause drowsiness, headache, and irritation of respiratory tract; Irritation of skin Proprietary fragrance mixtures Benzyl acetate Bornan-3-one (camphor) 3,7-Dimethyll-2,6-octadienal (citral)

CAS chemical abstracts service registry number, *MSDS* materials safety data sheet

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.

3.3 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 had undergone successful germination had protrusion of radicles that varied in length. Seedlings possessed a radicle with root hairs, hypocotyl, and a pair of green cotyledons.

Percent seed germination and seedling formation for the 14 VOC treatments at 1 ppm for 72 hours are presented in Figure 3. For controls, 96% of the seeds progressed to seedling while the rate of germination rate of exposed seeds was lower for all treatments. Approximately 35% of the seeds exposed to 2-octanol and 3-octanone did not germinate. The percentage of non-germinated seeds was nearly 50% for 1-octanol, over 60% for 1-octen-3-ol and 99% for 1-octen-3-one. 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, 90% percent of seeds successfully formed a radical, but almost none progressed to the seedling stage. The mold VOCs were all more effective in suppressing seed germination and seedling formation than the three solvents tested. 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%). The response of seeds exposed to 1 ppm of the fragrance mixtures was varied. Over 70% of the seeds exposed to 1 ppm of the air fresher 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 air freshener compound tested, where 58% had incomplete germination. No seeds exposed to scented oil progressed to seedling formation. 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). The inhibition of germination was most pronounced for the biogenic mold-related VOCs.

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 incubated for three additional days. Subsequent recovery and seedling formation were 95% for all treatments (data not shown).

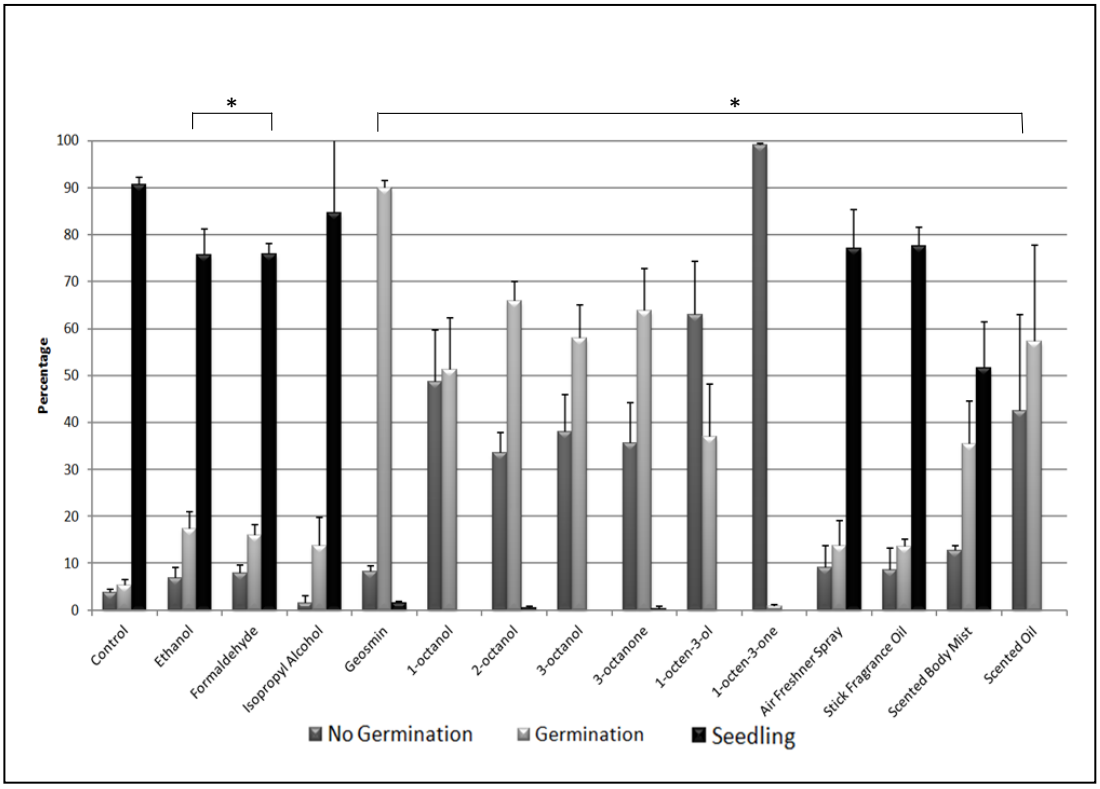


Figure 3. *Arabidopsis thaliana* exposed to VOCs at 1 ppm for 72 hours. Average percentage of seed germination and seedling development. Treatment was replicated three times and the experiment was repeated four times (n = 30). Error bars represent the standard deviation of the mean, where ANOVA P = 0.0001. * denotes significance.

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. Plants exposed to ethanol, isopropanol, and the four 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 reduced root size, bleached leaves, and did not resume growth after removal from the exposure conditions (Fig. 4A).

Plants exposed to 1 ppm of geosmin and the six C8 hydrocarbons exhibited a wide range of morphological changes compared to controls (Figure 4B). In addition to being smaller in size, many of these test plants had leaves that curled in as well as reduced root structures. Plants exposed to 2-octanol exhibited localized cell death with large sections of the leaves showing yellow and white discoloration. 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 tested, 1-octen-3-one, caused bleaching and death in plants after 72 hours of exposure (Fig. 4B).

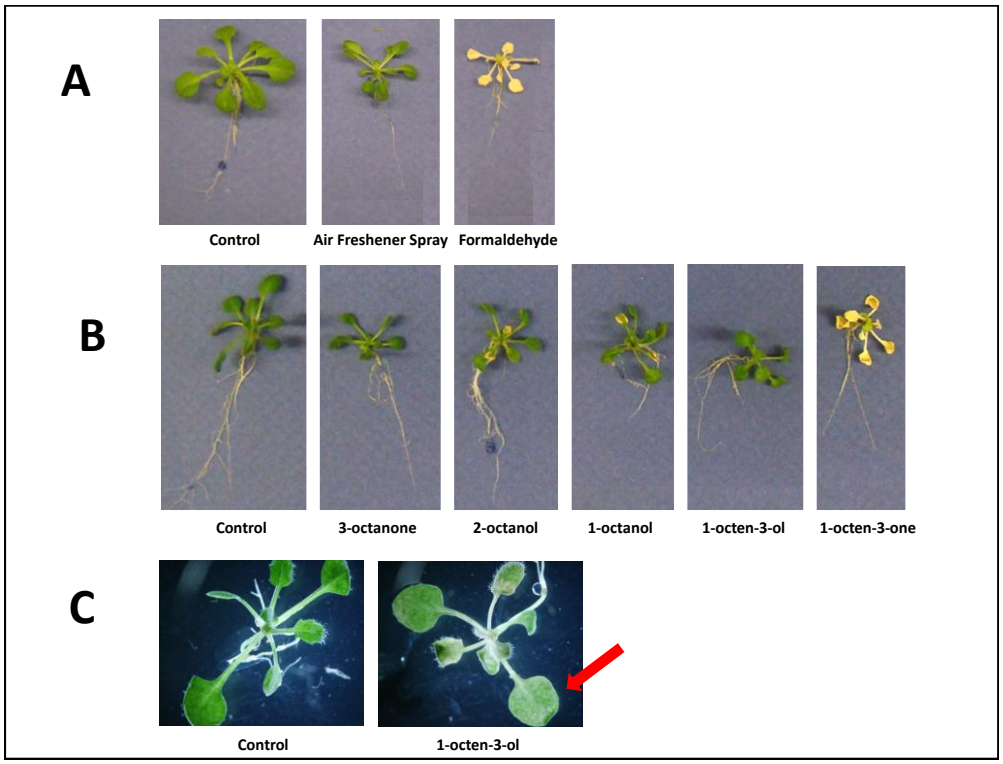


Figure 4. Fourteen-day-old *Arabidopsis thaliana* exposed to VOCs at 1 ppm for 72 hours. Visible indicators of plant stress to VOCs include smaller plant and leaf size, discoloration of leaves, small necrotic lesions, and complete death of plant. (A) Plants exposed to common anthropogenic VOCs compared 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).

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 chlorophyll concentration in plants exposed to common mold 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, ranging from 1.12 to 1.3±0.1 mg/g (a 32 and 41% decrease, respectively). Of the solvents tested, formaldehyde treatment resulted in the lowest chlorophyll concentration of 0.5±0.08 mg/g (a 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 treated plants had significantly less chlorophyll than did control plants (Student's t-tests, $P < 0.05$; ANOVA, $P = 0.0001$).

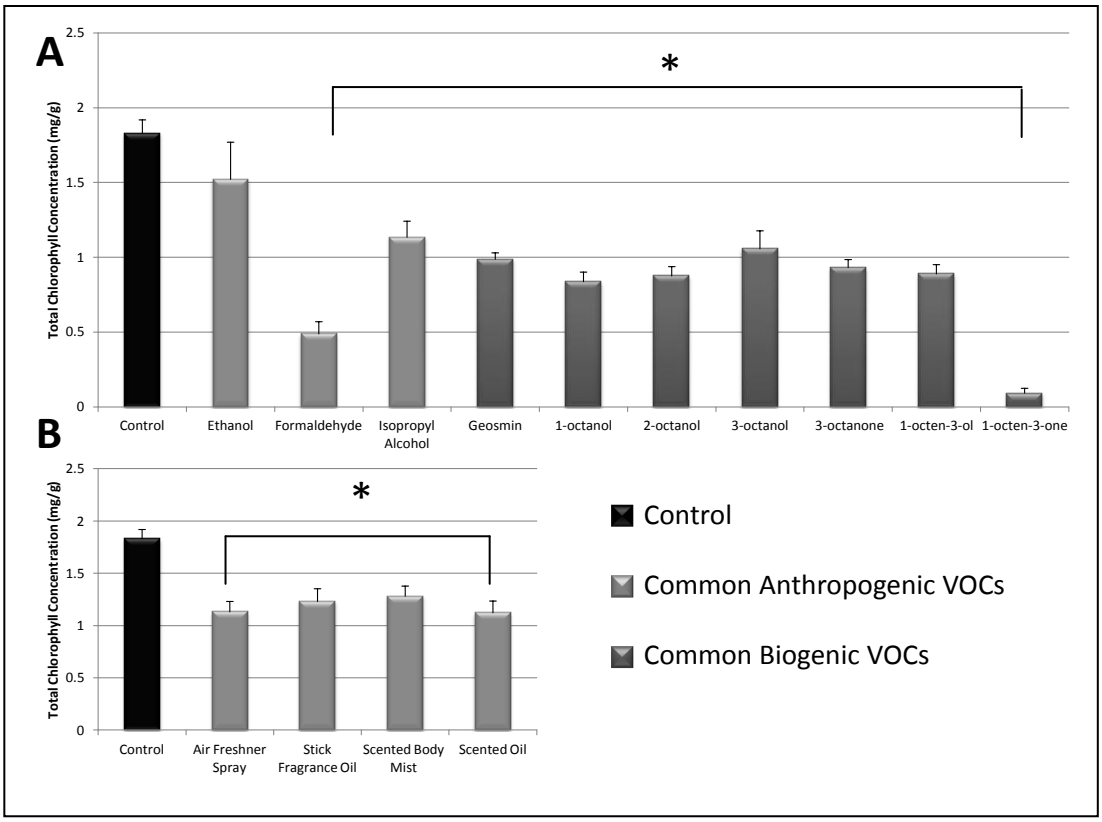


Figure 5. Total chlorophyll concentration of *Arabidopsis thaliana* exposed to VOCs at 1 ppm for 72 hours. (A) Comparison of all single VOC compounds. (B) Comparison of anthropogenic fragrance VOCs in mixtures. Treatment was replicated three times and the experiment was repeated three times (n = 12). Error bars represent the standard deviation of the mean (ANOVA, $P = 0.0001$). * denotes significance.

3.4 Discussion

The process of germination begins with the uptake of water and rupturing of the testa and is considered complete with the emergence of the radicle. Seedling vigor is assessed by evaluating the growth of the radicle followed by the emergence of hypocotyl and the cotyledons (Rajjou et al., 2012). We 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 and Monteiro, 2005).

The antagonistic activities of various volatile phase terpenoids, ketones, alcohols and aldehydes on seed germination and seedling formation have been studied in lettuce (Vokou et al., 2003), as well as in carrot, onion and tomato seeds (Bradow, 1991). Moreover, there have been a few previous studies in which *A. thaliana* was used to model possible negative effects of volatile phase molecules. Volatile-mediated killing of *A. thaliana* by bacteria was shown to be caused largely by hydrogen cyanide (Blom et al., 2011). 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). Splivallo et al. (2007) did not report significant effects at 1.3 ppm of 3-octanol, 3-octanone, trans-2-octenol, and several other volatiles emitted by truffles on primary root or cotyledon formation in *A. thaliana*. In these studies, compounds were diluted in solvents and the experimental set up differed from ours. In contrast, we observed inhibitory effects with undiluted compounds at 1 ppm for several of these C8 compounds. Exposure to 1-octen-3-one caused complete inhibition of germination while

1-octanol, 2-octanol, 3-octanol, 3-octanone, 1-octen-3-ol caused radicles to stop growing after they emerged and inhibited seedling formation. The inhibitory effects we observed were not lethal. Once removed from the presence of 1-octen-3-one and other C8 VOCs, seeds germinated and grew into seedlings. It is probable that Spivallo et al. (2007) did not make as strong a distinction between radicle emergence and seeding formation as we did, thereby explaining the difference between our studies.

Geosmin is a well-known biogenic volatile with an extremely low odor threshold (150 - 200 ng/m^3). 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 our 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 our studies, geosmin-exposed *A. thaliana* 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_3. In our 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 phytotoxicity 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 fungal 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 known to be 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 our study, seed germination and seedling formation were not inhibited by 1 ppm of formaldehyde.

Localized cell death in plant tissue is indicative of oxidative bursts caused by overabundance of ROS as a response to stress (Heller and 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 and Sunkar, 2005; Anjum et al., 2011). Drought, salinity, metal and temperature stresses imposed on vegetative stages cause similar effects (Jaleel et al., 2007; Mafakheri et al., 2010). Indicators of plant stress in our study included discoloration and curling of leaves, and localized necrotic lesions on leaf tissue. With the exception of ethanol, when compared to controls, all the VOCs treatments we studied gave rise to significantly lower levels of chlorophyll in exposed

vegetative 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; Mafakheri et al., 2010; Splivallo et al., 2007). Relatively short-lived, hydrogen peroxide is one of the earliest cytologically detectable defense responses of plant cell walls (Thordal-Christensen et al., 1997).

Of 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, 1-octen-3-one-exposed plants showed 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). Our 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, as well as commercial air freshener products, should receive more study in future toxicological testing of flavor and fragrance products. Many people use fragrance products containing both synthetic and biogenic VOCs to mask the odor of moldy basements, attics and other indoor environments. Such air fresheners products 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). For

example, although no toxicity tests were conducted, a study by Steineman et al. (2010) using headspace analysis with gas chromatography/mass spectrometry (GC/MS) showed that 25 fragranced products emitted 133 different VOCs, of which 24 were classified as toxic or hazardous under US Federal laws.

In conclusion, we have demonstrated the sensitivity of germinating seeds, and two week old plants of *A. thaliana,* to the presence of low concentration of a variety of VOCs characteristic of molds, common solvents and commercial air freshener products. The concentration used in this study is higher than those encountered in most natural environments but lower than those used in previously published studies that ranged from 1.3-130 ppm (Ogura, 2000; Splivallo et al., 2007). Our studies show that *A. thaliana* provides a useful, rapid model for testing phytotoxicity and that results of plant phytotoxicity tests for mold VOCs yield support those previously determined by more expensive tissue culture and animal studies (Kreja and Seidel, 2002; Korpi et al., 2009; Inamdar et al., 2011). We suggest that *A. thaliana* can be used in preliminary screens to determine the possible adverse health effects of other biogenic and anthropogenic VOCs. Furthermore, in addition to serving as a useful bioassay, the wealth of genetic information and available mutants stocks for *A. thaliana* will facilitate the analysis of VOC phytotoxicity at the molecular level.

REFERENCES

Almeras, E., Stolz, S., Vollenweider, S., Reymond, P., Mene-Saffrane, L., Farmer, E.E. (2003). Reactive electrophile species activate defense gene expression in *Arabidopsis*. The Plant Journal. 34: 202-216.

Anjum, S.A., Xie, X., Wang, L., Salee, M.F., Man, C., Lei, W. (2011). Morphological, physiological and biochemical responses of plants to drought stress. African Journal of Agricultural Research. 6: 2026-2032.

Araujo, A.S.F., Monteiro, R.T.R. (2005). Plant bioassays to assess toxicity of textile sludge compost. Scientia Agricola. 62: 286-290.

Baechler, M.D., Hadley, D.L., Marseille, T.J., Berry, M.A. (1991). Sick building syndrome. Sources, health effects, mitigation. Noyes Data Corporation, New Jersey.

Bartels, D., Sunkar, R. (2005). Drought and salt tolerance in plants. Critical Reviews in Plant Sciences. 24: 23-58.

Banks, M.K., Schultz, K.E. (2005). Comparison of plants for germination toxicity tests in petroleum-contaminated soils. Water, Air, and Soil Pollution. 167: 211-219.

Berger, R.G. (Ed.) (2007). Flavours and Fragrances – Chemistry, Bioprocessing 379 and Sustainability. Springer-Verlag.

Bhattacharjee, S. (2012). The language of reactive oxygen species signaling in plants. Journal of Botany. doi:10.1155/2012/985298.

Blom, D., Fabbri, C., Eberl, L., Weisskopf, L. (2011). Volatile-mediated killing of *Arabidopsis thaliana* by bacteria is mainly due to hydrogen cyanide. Applied and Environmental Microbiology. 77: 1000-1008.

Bradow, J.M. (1991). Relationships between chemical structure and inhibitory activity of C6 through C9 volatiles emitted by plant residues. Journal of Chemical Ecology. 17: 2193 - 2198.

Cape, J.N. (2003). Effects of airborne volatile organic compounds on plants. Environmental Pollution. 122: 145-157.

Ebi, K.L., McGregor, G. (2008). Climate change, tropospheric ozone and particulate matter, and health impacts. Environmental Health Perspectives. 116: 1449-1455.

Environmental Protection Agency. (1994). US Environmental Protection Agency Catalogue of Standard Toxicity Tests for Ecological Risk Assessment Publication 9345.0-05I Intermittent Bulletin Volume 2, Number 2.

Gutiérrez-Luna, F.M., López-Bucio, J., Altamirano-Hernández, J., Valencia-Cantero, E., de la Cruz, H.R., Macías-Rodríguez, L. (2010). Plant growth-promoting rhizobacteria modulate root-system architecture in *Arabidopsis thaliana* through volatile organic compound emission. Symbiosis. 51: 75-83.

Heller, J., Tudzynski, P. (2011). Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annual Review of Phytopathology. 49: 369-390.

Herrmann, A. (Ed.) (2010). The Chemistry and Biology of Volatiles. JohnWiley & Sons.

Hung, R., Lee, S., Bennett, J.W. (2013). *Arabidopsis thaliana* as a model system for testing the effects of *Trichoderma* volatile organic compounds. Fungal Ecology. 6: 19-26.

Inamdar, A.A., Moore, J.C., Cohen, R.I., Bennett, J.W. (2011). A model to evaluate 424 the cytotoxicity of the fungal volatile organic compound 1-octen-3-ol in human embryonic stem cells. Mycopathologia. 173: 13-20.

Jaleel, C.A., Sankar, B., Sridharan, R., Panneerselvam, R. (2007). Soil salinity alters growth, chlorophyll content, and secondary metabolite accumulation in *Catharanthus roseus*. Turkish Journal of Biology. 32: 79-83.

Jiang, J., He, X., Cane, D. (2007). Biosynthesis of the earthy oderant geosmin by a bifunctional streptomyces coelicolor enzyme. Nature Chemical Biology. 3: 711-715.

Juang, D.F., Lee, C.H., Chen, W.C., Yuan, C.S. (2010). Do the VOCs that evaporate from a heavily polluted river threaten the health of riparian residents? Science of the Total Environment. 408: 4524-4531.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J. (2007). Volatile 1-octen-3-ol induces a defensive response in *Arabidopsis thaliana*. Journal of Plant Pathology. 73: 35-37.

Korpi, A., Jarnberg, J., Pasanen, A.L. (2009). Microbial volatile organic compounds. Critical Reviews in Toxicology. 39: 139-193.

Kreja, L., Seidel, H.J. (2002). On the cytotoxicity of some microbial volatile organic compounds as studied in the human lung cell line A549. Chemosphere. 49: 105-110.

Li, D.W., Yang, C.S. (2004). Fungal contamination as a major contributor to sick building syndrome. Advances in Applied Microbiology. 55: 31-112.

Mafakheri, A., Siosemardeh, A., Bahramnejad, B., Struik, P.C., Sohrabi, E. (2010). Effect of drought stress on yield, proline and chlorophyll contents in three chickpea cultivars. Australian Journal of Crop Science. 4: 580-585.

Mølhave, L. (2009). Volatile organic compounds and the sick building syndrome. In: Lippmann, M. (Ed.) Environmental Toxicants: Human Exposures and their health effects. Wiley-Interscience. Pgs. 241-256.

Mutters, R.G., Madore, M., Bytnerowicz, A. (1993). Formaldehyde exposure affects growth and metabolism of common bean. Journal of the Air & Waste Management Association. 43: 113-116.

Nikolaou, A.D., Golfinopoulos, S.K., Kostopoulou, M.N., Kolokythas, G.A., Lekkas, T.D. (2002). Determination of volatile organic compounds in surface waters and treated wastewater in Greece. Water Research. 36: 2883-2890.

Ogura, T., Sunairi, M., Nakajima, M. (2000). 2-Methylisoborneol and geosmin, the main sources of soil odor, inhibit the germination of Brassicaceae seeds. Soil Science. 46: 217-227.

Ommen, O.E., Donnelly, A., Vanhoutvin, S., van Oijen, M., Manderscheid, R. (1999). Chlorophyll content of spring wheat flag leaves grown under elevated CO_2 concentrations and other environmental stresses within the ESPACE-wheat project. European Journal of Agronomy. 10: 197-203.

Ortiz-Castro, R., Contreras-Cornejo, H.A., Macias-Rodriguez, L., Lopez-Bucio, J. (2009). The role of microbial signals in plant growth and development. Plant Signaling & Behavior. 4: 701-712.

Potera, C. (2011). Indoor air quality: scented products emit a bouquet of VOCs. Environmental Health Perspectives. 119:A16. doi: 10.1289/ehp.119-a16.

Rajjou, L., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C., Job, D. (2012). Seed germination and vigor. Annual Review of Plant Biology. 63: 507-533.

Splivallo, R., Fischer, U., Göbel, C., Feussner, I., Karlovsky, P. (2009). Truffles regulate plant root morphogenesis via the production of auxin and ethylene. Plant Physiology. 150: 2018-2029.

Splivallo, R., Novero, M., Bertea, C.M., Bossi, S., Bonfante, P. (2007). Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist. 175: 417-424.

Steinemann, A.C., MacGregor, I.C., Gordon, S.M., Gallagher, L.G., Davis, A.L., Ribeiro, D.S., Wallace, L.A. (2010). Fragranced consumer products: chemicals emitted, ingredients unlisted. Environmental Impact Assessment Review. doi:10.1016/j.eiar.2010 .08.002.

Steiner, A.L., Tonse, S., Cohen, R.C., Goldstein, A.H., Harley, R.A. (2006). Influence of future climate and emissions on regional air quality in California. Journal of Geophysical Research. 111: D18303. doi:10.1029/2005JD006935.

Stensmyr, M.C., Dweck, H.K.M., Farhan, A., Ibba, I., Strutz, A., Mukunda, L., Linz, J., Grabe, V., Steck, K., Lavista-Llanos, S., Wicher, D., Sachse, S., Knaden, M., Becher, P.G., Seki, Y., Hansson, B.S. (2012). A conserved dedicated olfactory circuit for detecting harmful microbes in *Drosophila*. Cell. 151: 1345-1357.

Straus, D.C. (2004). Sick building syndrome. In: Advances in Applied Microbiology Vol. 55. Elsevier Academic Press.

Takigawa, T., Wang, B.L., Saijo, Y., Morimoto, K., Nakayama, K., Tanaka, M., Shibata, E., Yoshimura, T., Chikara, H., Ogino, K. (2009). Relationship between indoor chemical concentrations and subjective symptoms associated with sick building syndrome in newly built 516 houses in Japan. International Archives of Occupational and Environmental Health. 83: 225-235.

Thordal-Christensen, H., Zhang, Z., Wei, Y., Collinge, D.B. (1997). Subcellular localizastion of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-mildew interaction. The Plant Journal. 11: 1187-1194.

Wang, X., Sun, C., Gao, S., Wang, L., Shuokui, H. (2001). Validation of germination rate and root elongation as indicator to assess phytotoxicity with *Cucumis sativus*. Chemosphere. 44: 1711-1721.

Vokou, D., Douvli, P., Blionis, G.J., Halley, J.M. (2003). Effects of monoterpenoids, acting alone or in pairs, on seed germination and subsequent seedling growth. Journal of Chemical Ecology. 29: 2281-2301.

Walinder, R., Ernstgard, L., Johanson, G., Norback, D., Venge, P., Wieslander, G. (2005). Acute effects of a fungal volatile compound. Environmental Health Perspectives. 113: 1775-1778.

Watson, S. (2003). Cyanobacterial and eukaryotic algal odour compounds: signals or by-products? A review of their biological activity. Phycologia. 42: 332-350.

World Health Organization. (2009). Guidelines for indoor air quality: dampness and mold. Druckpartner Moser, Germany.

Wilkins, K., Larsen, K., Simkus, M. (2000). Volatile metabolites from mold growth on building materials and synthetic media. Chemosphere. 41: 437-446.

Zogorski, J.S., Carter, J.M., Ivahnenko, T., Lapham, W.W., Moran, M.J., Rowe, B.L., Squillace, P.J., Toccalino, P.L. (2006). The quality of our nation's waters--volatile

organic compounds in the nation's ground water and drinking-water supply wells. US Geological Survey Circular. 1292:101.

CHAPTER 4.

Analysis of Volatile Organic Compounds Emitted by *Trichoderma* species and Volatile-Mediated Plant Growth

The author is currently preparing a manuscript with results from this chapter which will be submitted for publication.

4.1 Introduction

The genus *Trichoderma* is one of the most widely researched genera of filamentous fungi with numerous applications in agriculture, industry and the environment (Schuster and Schmol, 2010; Mukherjee et al., 2013). Several *Trichoderma* species are used extensively for the production of industrial enzymes. For example, *T. reesei* is studied extensively for the production of cellulolytic and hemicellulolytic enzymes (Kubicek, 2013). *T. inhamatum*, *T. viride*, and a few additional species have demonstrated potential applications in the bioremediation of wastes including metals in soil (Bishnoi et al., 2007; Morales-Barrera and Cristiani-Urbina, 2008; Tripathi et al., 2013). In agriculture, *Trichoderma* has the ability to reduce plant diseases and promote plant growth and productivity (Harman et al., 2004; Ortiz-Castro et al., 2009; Szabo et al., 2012). *Trichoderma* species are robust biological control agents because they utilize several modes of action including resistance, antibiosis, competition and myco-parasitism (Whipps and Lumsden, 1989). Moreover, since *Trichoderma* species possess innate resistance to many chemicals used in agriculture such as fungicides, they are readily integrated into pest management practices (Chaparro et al., 2011).

Trichoderma species are prolific producers of many small metabolites with medical and agricultural significance (Mathivanan et al., 2008; Mukherjee et al., 2012). *Trichoderma* produces non-volatile metabolites such as peptaibols and polyketides. These compounds often exhibit antifungal, antibacterial, and anticancer properties. They also can induce resistance to pathogens in plants or they are produced as toxins (Engelberth et al., 2001; Mukherjee et al., 2012). Volatile metabolites, also known as volatile organic compounds (VOCs), have low molecular mass, high vapor pressure (>0.01 k Pa), low boiling point, and low polarity (Insam and Seewald, 2010). They are

chemically diverse and include hydrocarbons, aromatic compounds, and terpenes of these, only the terpenes are secondary metabolites.

Although VOCs are a smaller portion of the total metabolites (intermediate and end products) produced by organisms, their unique properties enable them to mediate important biological functions, especially in aerial and terrestrial environments. There exists a large body of literature investigating volatiles and their roles as semio- or info-chemicals for insects and microorganisms. VOCs in mixtures and single compounds are able to induce a wide range of inhibitory or beneficial effects in biological systems (Korpi et al., 2009; Insam and Seewald, 2010; Morath et al., 2012; Bitas et al., 2013; Penuelas et al., 2014; Hung et al., 2015; Kanchiswamy et al., 2015).

In agriculture, fungal VOCs have been used to suppress pathogenic bacteria and fungi (Campos et al., 2010). In a process called "mycofumigation", the VOCs from endophytic fungi in the genus *Muscodor* are able to kill several pathogenic fungi and bacteria (Strobel et al., 2001; Mitchell et al., 2010; Kudalkar et al., 2012). The antimicrobial activities of *Trichoderma* VOCs have been documented early on (Dennis and Webster, 1971). Several species of *Trichoderma* (*T. harzianum*, *T. viride*, *T. lignorum*, *T. hamatum*, *T. pseudokoningii*, *T. reesei*) produce VOCs that inhibit the growth of phytopathogens and wood decay fungi (Wheatley et al., 1997; Aguero et al., 2008; Chakraborty and Chatterjee, 2008; Raza et al., 2013; Zhang et al., 2014; Aarti and Meenu, 2015).

In recent years, the use of *Trichoderma*-produced VOCs to mediate plant growth has been explored. One of the earliest volatile compounds to be identified from *Trichoderma* was the coconut odor, 6-pentyl-2H-pyran-2-one (6PP) (Collins and Halim,

1972). Treating plants with a low concentration (0.166 - 1 mg/l) of 6PP induced growth promotion and reduced disease symptoms (Vinale et al., 2008). Previously, we reported the ability of *Trichoderma* to stimulate plant growth by emitting naturally occurring mixtures of VOCs. Plants exposed to *Trichoderma*-derived VOCs improved plant growth by increasing plant size, fresh weight, chlorophyll, root growth, and number of flowers (Hung et al., 2013). *Trichoderma* volatile-mediated plant growth promotion was dependent on the age of the fungal culture, developmental stage of the plant, duration of the exposure, and was isolate-specific (Hung et al., 2013; Lee et al., 2015).

Most of the studies on *Trichoderma* volatiles have been conducted on a few select species important as biological control agents, and focused primarily on their effects on inhibition of pathogenic microbes or induction of plant systemic resistance. Our study focused on plant growth promotion in the absence of pathogen threat. We hypothesized that since volatile-induced plant growth promotion is isolate or strain specific, we expect to see variations in the beneficial plant response to *Trichoderma* VOCs. Our goal was to find the optimal plant growth promoting volatile producing isolate from a broad collection of *Trichoderma* species and to characterize isolate specific volatile profiles.

In this study, we assessed 20 *Trichoderma* samples for their volatile production and effects on plant growth and development. We first screened *Trichoderma* isolates for VOC-induced growth using the model plant, *Arabidopsis thaliana*. Isolate that stimulated growth in *Arabidopsis* was further screened using *Solanum lycopersicum* (tomato) seedlings. Finally, complete *Trichoderma* volatile profiles for isolates were identified using gas chromatography-mass spectrometry (GC-MS) analysis.

4.2 Materials and methods

Cultivation fungal isolates

Trichoderma isolates were obtained from Dr. Amy Y. Rossman at USDA-ARS, Beltsville, MD. See Table 1 for isolate, culture collection and collector information. Cultures were maintained on potato dextrose agar (PDA) and malt extract agar (MEA) (Difco) containing Petri plates and maintained at 27±2°C in the dark with > 80% humidity. Prior to volatile-exposure bioassay, the fungus was grown in 35x10 mm Petri dish on 4 ml of MEA and incubated for 5 days at 27±2°C.

Plant growth conditions

Arabidopsis thaliana seeds (ecotype Columbia-7) were obtained from the *Arabidopsis* Biological Resource Center (Columbus, OH). The seeds were surface-sterilized in 95 % ethanol for 30 sec followed by a 20 % bleach solution for 30 min with constant agitation. Five surface-sterilized seeds were sown onto a 100 × 15 mm partitioned Petri dish (also known as split or I-plate) or 60 x 15 mm Petri dish containing Murashige and Skoog (MS) medium with vitamins, 3 % sucrose, and 0.03 % phytagel (pH 5.7) (Phytotechnology Laboratories, KS). Seeds were stratified at 4 °C for three days prior to volatile exposure.

Seeds of tomato (*Solanum lycopersicum* L. cv. Ponderosa) were purchased commercially. Seeds were surface sterilized with 70% ethanol for 30 sec and a 15% bleach solution for 20 min with constant agitation. Prior to the exposure assay, two surface-sterilized seeds were sown onto a 473 ml volume sterile culture vessel (SteriCon, PhytoTechnology Laboratories, KS) containing 100 ml of MS media (1% sucrose, 0.03% phytagel, and pH 5.7).

***Trichoderma* species**	**Location**	**Source**
T. aggressivum (DAOM222156)	Ontario, Canada	Mushroom casing
T. aggressivum (IMI 393970)	PA, USA	Mushroom compost
T. asperellum (CBS 433.97)	Beltsville, MD, USA	Soil, sclerotia buried in sesame plot
T. asperellum (GJS 02-65)	Douala, Loum, Cameroon	Soil, *Xanthostoma sagittifolium* roots and soil
T. atroviride (CBS 351.93)	NC, USA	Soil, forest
T. atroviride (GJS 01-209)	Cameroon	Palm
T. atroviride (JWB)	New Orleans, LA, USA	Building, Hurricane Katrina damaged
T. brevicompactum (CBS 109720)	NY, USA	Soil, under Helianthus
T. harzianum(CBS 226.95)	United Kingdom	Soil
T. harzianum(CBS 227.95)	United Kingdom	Soil
T. inhamantum (CBS 273.78)	Colombia	Soil, maize field
H. koningii (CBS 989.97)	MD, USA	Decorticated wood (*T. koningii* type specimen)
T. longibrachiatum (CBS 118642)	Mexico	Soil
T. longibrachiatum (TR97)	OH, USA	Soil
T. pseudokoningii (CBS 480.91)	Australia	Wood, decayed
T. pseudokoningii (CBS 130756)	Australia	Wood, decorticated
T. stromaticum (GJS 00-127)	Bahia, Brazil	Theobroma cacao, pod
T. virens (DAOM167651)	GA, USA	Soil, cultivated
T. viride (BBA 70239)	Denmark	Building, water damaged
T. viride (GJS 04-379)	Brazil	Soil

Table 1. *Trichoderma* isolates screened for volatile-induced growth promotion. Abbreviations of culture collections and collectors as follows: BBA = Biologisches Bundesanstalt, Berlin, Germany; CBS = Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; DAOM = Canadian Collection of Fungal Cultures, Ottawa, Canada; GJS = Gary J. Samuels collection (Culture collection of the United States Department of Agriculture, Systematic Botany and Mycology Lab, Beltsville, MD, U.S.A.); IMI InternationalMycologial Institute (New Zealand); JWB = Joan W. Bennett collection (Rutgers, New Brunswick, NJ, U.S.A.); TR = Earl Nelson collection (USDA-ARS, Beltsville)

Plant-Trichoderma volatile-exposure bioassay

Experiments were completed using a double plate-within-a-plate system used for *Arabidopsis* exposure to *Trichoderma* VOCs according to previously described methods (Lee et al., 2015). Briefly, a small Petri plate (35 × 10 mm) containing sporulating *Trichoderma* grown on MEA was placed into a larger partitioned Petri dish (100 x 15

mm) containing five stratified *A. thaliana* seeds. Plants and fungi were grown together in a growth chamber with a 16-hour photoperiod at 23 ± 1 °C, 45% relative humidity, and 180 µmol m^{-2} s^{-1} light for 14 days. For controls, plants were exposed to the fungal growth medium alone.

To expose tomato seeds, a plate (35 × 10 mm) containing the *Trichoderma* culture was placed inside a sterile foil container (50 ml volume). The foil containing the fungal plate was then placed inside the culture vessel containing sterilized seeds. Tomato seeds were germinated in the presence of *Trichoderma* VOCs and grown together in a growth chamber with 16-hour photoperiod at 25±1°C for 21 days. At the end of the exposure period, the *Arabidopsis* and *Solanum* plants were removed from the exposure conditions, photographed, and the fresh weight of plant shoots and total chlorophyll content were measured.

Total chlorophyll content of plants exposed to *Trichoderma* VOCs was determined by submerging the shoot overnight in 1 ml of 80 % acetone in the dark at 4 °C. The total chlorophyll content (chlorophyll a and b) was calculated from the equation $[(8.02)(A663) + (20.2)(A645)]V/1000\ W$, where V is volume and W is plant fresh weight. The chlorophyll data were expressed in relation to the fresh weight of the plant shoot.

Five replicates were used per volatile exposure condition, and the experiments were repeated three times. Quantitative results were expressed as standard error of the mean and analyzed using R Statistical Software (version 3.2.1, World Famous Astronaut). One-way analysis of variance (ANOVA) and Student's t-test between groups was performed for plant exposure quantitative data.

VOC Analysis by Headspace GC-MS

For headspace volatile analysis, *Trichoderma* cultures were grown in a 500 ml glass flask containing 250 ml of MEA with a 16-hour photoperiod at 27±2°C for 7 days. VOC capture and analysis were conducted as described previously using a purge and trap method (Lee et al., 2015). Headspace samples taken from sterile MEA served as negative controls. The headspace of the flask was purged at 100 ml per min for four hours. The VOCs were adsorbed on 6 cm Tenax columns (Scientific Instrument Services, Ringoes, NJ), 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 (SigmaAldrich Corp., St. Louis, MO) column: 0.32 mm diameter, 1 mm film thickness. The compounds 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: 10:1 split, helium carrier at 20 psi, oven temperature from -20 ˚C to 280 ˚C at 10 ˚C per min. The MS conditions were: positive ion mode, electron impact spectra at 70 eV. The MS of the peaks were determined by their scatter pattern. Internal standards (d-6 benzene, d-8 toluene, and d-8 napthalene) were used to normalize the peak areas. The linear regression coefficient was used to calculate the concentrations in the samples from peak areas obtained in the chromatographs. Compounds were identified by comparison of spectra obtained from the *Trichoderma* samples with those from a reference library (NIST 08 Mass Spectra Library, National Institute of Standards and Technology). GC-MS analysis was completed in triplicates per isolate studied.

The concentration of individual compounds determined through the GC-MS analysis was further analyzed and visualized using R Statistical Software (version 3.1.2,

"Pumpkin Helmet"). One-way analysis of variance (ANOVA) followed by post hoc test, Tukey's honestly significant difference (HSD) test was completed on quantitative data. The following pairwise statistical tests were completed between isolates belonging to same species: Student's t-test, Welch's t-test, and Wilcoxon-Mann-Whitney test. The two latter tests were employed to re-verify the finding of hypothesis rejection at $p<0.05$. The Welch's t-test accounted for unequal variance and the Wilcoxon–Mann–Whitney test accounted for non-normally distributed (non-parametric) data. A heatmap was generated using "heatmap.2" (gplots package) for presence and absence of compounds detected in triplicates per sample tested.

4.3 Results

Isolate specific volatile-mediated plant growth promotion

Twenty different *Trichoderma* isolates (Table 1) were screened for their volatile-mediated effects on *Arabidopsis* Col-7 plants. *Arabidopsis* grown in the presence of *Trichoderma* VOCs exhibited a range of effects from growth inhibition to increased plant size (Fig. 1). Plants exposed to nine growth promoting, stimulatory *Trichoderma* isolates were generally larger in size and appeared greener. One isolate appeared to produce inhibitory volatiles leading to a reduction in plant size and localized death in plant leaves (Fig. 1B). Half of the isolates screened in this study did not alter the growth of the plants under our test conditions.

Following 14 days of exposure, plants were collected, the fresh weight and the total chlorophyll content of plant shoots were measured (see Fig. 2). We defined an isolate as growth promoting if both the plant fresh shoot weight and total chlorophyll content were significantly higher than controls. We identified nine isolates that promoted *Arabidopsis* growth. The strongest volatile-mediated plant effects were observed in *T.*

aggressivum (IMI 393970) with an increase of 37.1% fresh shoot weight and 82.5% chlorophyll and *T. pseudokoningii* (CBS 130756) with 41.6% and 89.3% respectively. In contrast *T. atroviride* (CBS 01-209) emited inhibitory VOCs, leading to small plants with a 13.1% decrease in fresh shoot weight and 50.5% decrease in chlorophyll.

We measured CO_2 production by *Trichoderma* using a CO_2 monitor and did not find significant difference between CO_2 production by the fungi and ambient air in our testing conditions. In addition, trapping *Trichoderma*-derived CO_2 with 0.1 M KOH solution did not remove the observed volatile-induced beneficial effects (data not shown).

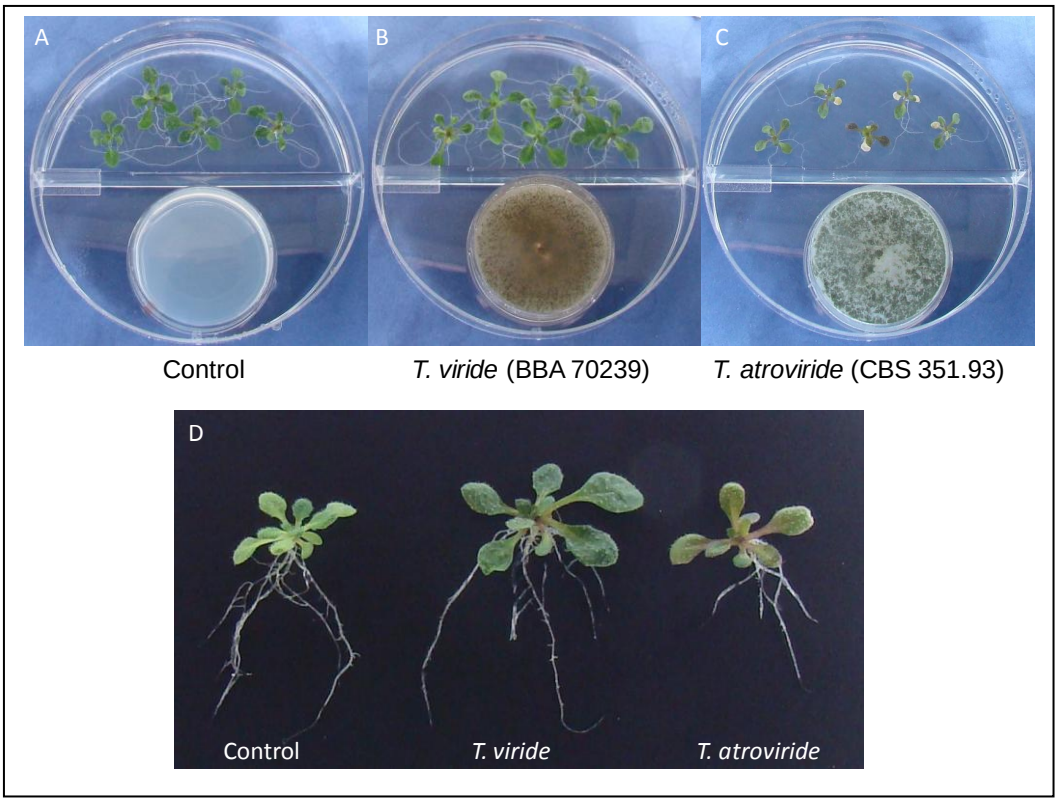


Figure 1. Growth of *Arabidopsis thaliana* in a shared atmosphere with *Trichoderma* for 14 days. (A) Control plants exposed to MEA medium, (B) plants exposed to *T. viride* (BBA 70239) are larger, (C) plants exposed to *T. atroviride* (CBS 351.93) are smaller, and (D) *Arabidopsis* plants removed from growth medium following 14-day *Trichoderma* volatile exposure.

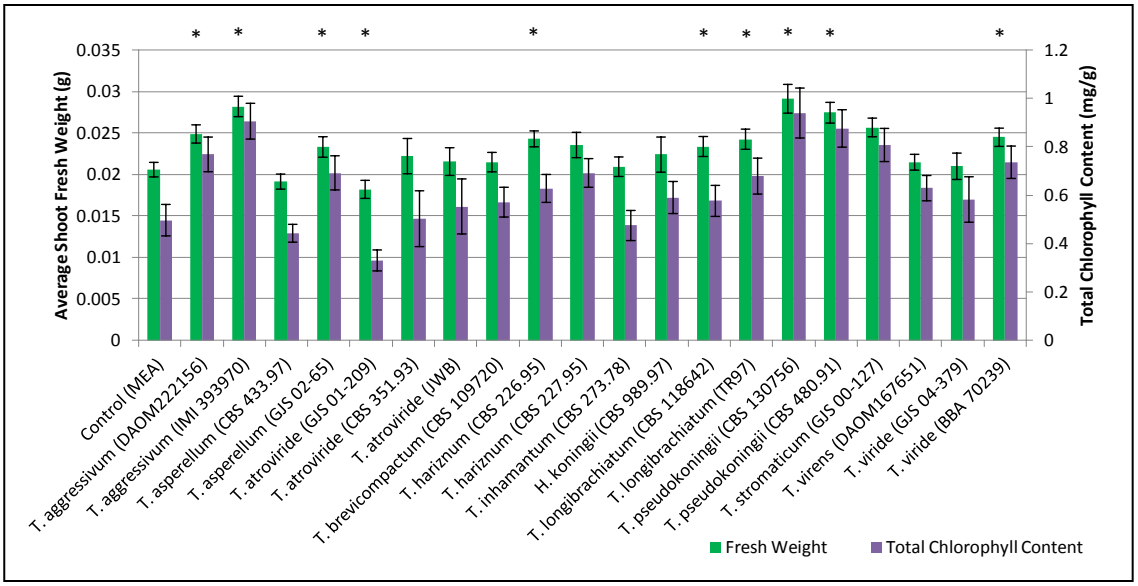


Figure 2. Average fresh weight and total chlorophyll content of *Arabidopsis thaliana* plants after growing in a shared atmosphere with 20 different isolates of *Trichoderma* for 14 days (n = 25, ANOVA P = 0.001).

The effects of Trichoderma volatiles on tomato shoot and root growths

We selected the *Arabidopsis* growth promoting isolate, *T. viride* (BBA 70239) and measured plant growth to assess its effects on tomato growth after 14 and 21 days of exposure. Tomato seedlings exposed to *T. viride* VOCs were larger in size (Fig. 3), with increases in the lateral root development (Fig. 3C). The fresh root weight was 61.2% greater than controls. Similarly, tomato plants exposed to *T. viride* VOCs for 14 days had a significant increase in biomass (41.2%) and chlorophyll concentration (70.7%). Extending the duration of volatile exposure to 21 days led to a larger increase in both tomato fresh weight (99.7%) and chlorophyll (100.1%).

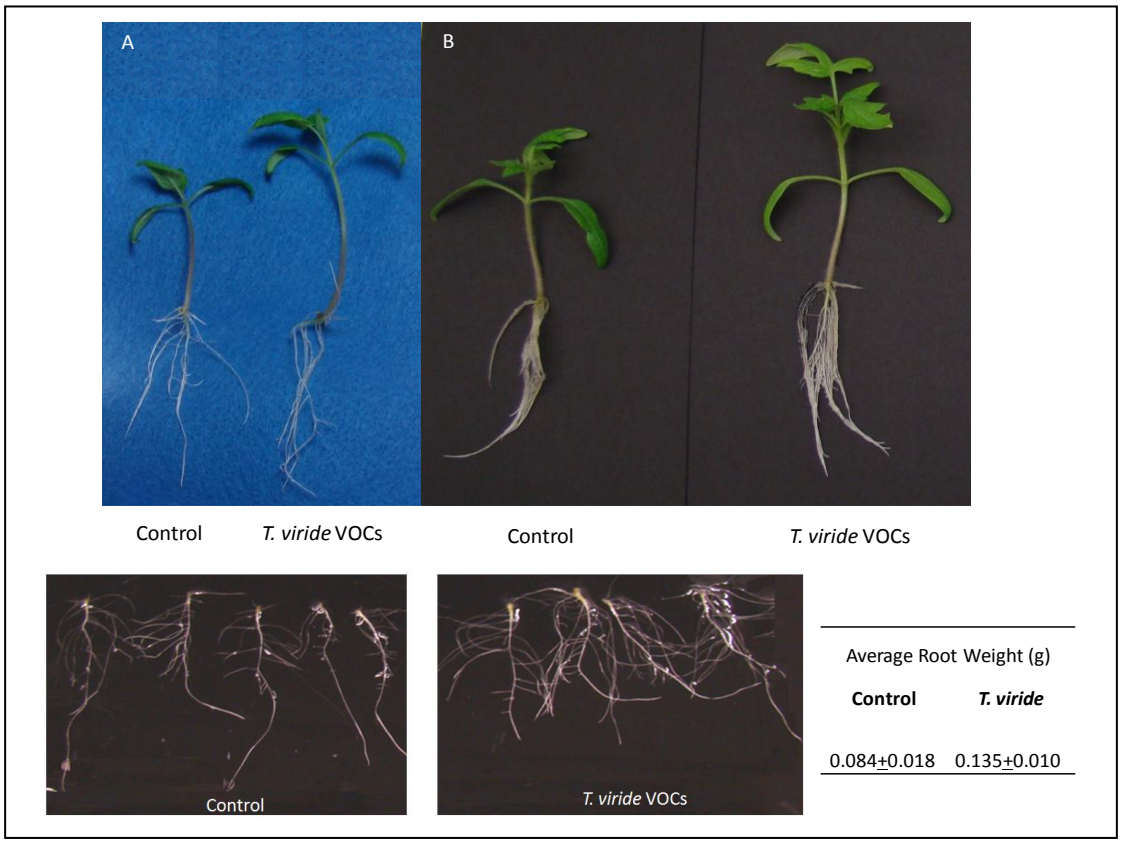


Figure 3. Tomato seedlings exposed to *T. viride* (BBA 70239) VOCs for (A) 14 days and (B) 21 days. (C) Roots of tomatoes exposed to *Trichoderma* VOCs for 21 days. (D) Average fresh root weight of tomato seedlings exposed to *Trichoderma* VOCs for 21 days.

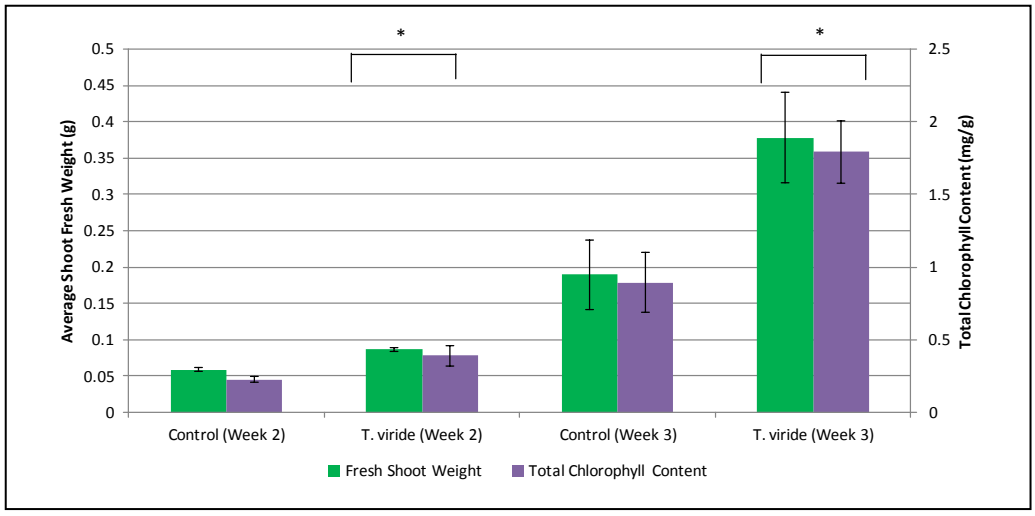


Figure 4. Shoot fresh weight and total chlorophyll content of tomato seedlings following 14- and 21-day-exposure to *T. viride* (BBA 70239) VOCs (n = 10, P = 0.01).

Identification of VOCs produced by Trichoderma

Nineteen of the *Trichoderma* isolates studied in the plant bioassay system was grown in MEA, the headspace collected for 4 hours then analyzed by GC-MS. The analysis was performed in triplicates. A total of 147 unique volatile compounds were detected at least twice per isolate (Table 2). They include a large number of C2 - C20 compounds including hydrocarbons, alcohols, ketones, aldehydes, alkanes, alkenes, esters, aromatic compounds, heterocyclic compounds, and various terpenes. C8 and C10 compounds were dominant, making up 17.01% and 15.64% respectively of all *Trichoderma* VOCs identified in this study. We also found several unknown 204, 222, 272, and 290 molecular weight monoterpenes, sesquiterpenes, and tetraterpenes (data not presented). We did not detect production of terpenes in control MEA headspace samples.

All *Trichoderma* isolates produced 3-methylbutanal, octanal, nonanal, and decanal, while acetoin (found in 18 isolates), 2-butanone (18), 3-methyl-1-butanol (17), 2-methyl-1-propanol (16), and acetone (16) were produced by most *Trichoderma* studied here. Several terpenes, limonene (18), β-caryophyllene (16), β-farnesene (14), and 2-norpinene (13) also were consistently found in *Trichoderma* isolates. One of the highest concentrations of volatile metabolite we identified in this study was 6-pentyl-2H-pyran-2-one (7559.45 ng/trap). However, this compound was found in a limited number of *Trichoderma* isolates, *T. aggressivum* (DAOM 222156 and IMI 393970), *T. atroviride* (GJS 01-209), *T. asperellum* (GJS 02-65), and *T. virens* (DAOM 167651). Several compounds were unique to specific *Trichoderma* isolates such as α- and β-phellandrene (*T. koningii* CJS 89-122), octadecane (*T. asperellum* GJS 02-65), trans-3-penten-2-ol (*T.*

harzianum CBS 227.95), 2-heptylfuran (*T. atroviride* JWB), 1-decene (*T. atroviride* JWB).

Chemical Name (alternate name)	RT (min)	Chemical Formula	MW (g/mol)	*Trichoderma* Isolates
ethanol	6.61	C2H6O	46.07	1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 16, 17, 19
propan-2-one (acetone)	6.85	C3H6O	58.08	1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19
2-methylpropanal (isobutyraldehyde)	7.43	C4H8O	72.11	1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19
propan-2-ol (2-propanol; isopropyl alcohol)	7.53	C3H8O	60.10	2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 17
methyl acetate	7.66	C3H6O2	74.08	2, 9, 13
butane-2,3-dione (diacetyl)	8.39	C4H6O2	86.09	3, 4, 5, 6, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19
butanal (butyraldehyde)	8.46	C4H8O	72.11	1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15
butan-2-one (2-butanone)	8.59	C4H8O	72.11	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
2-methylfuran	8.74	C5H6O	82.10	2
acetic acid	9.23	C2H4O2	60.05	3, 4, 5, 6, 9, 10, 13, 14, 15, 16, 17, 18, 19
propan-1-ol (propanol)	9.46	C3H8O	60.10	2, 11, 13
2-methylpropan-1-ol (2-methyl-1-propanol)	9.56	C4H10O	74.12	1, 2, 3, 4, 5, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19
ethyl acetate	9.59	C4H8O2	88.11	2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
3-methylbutanal	10.26	C5H10O	86.13	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
2-methylbutanal (2-methylbutyraldehyde)	11.17	C5H10O	86.13	1, 2, 4, 5, 8, 9, 10, 11, 12, 13, 14, 16, 17, 19
pentanal	11.34	C5H10O	86.13	1, 2, 4, 5, 7, 8, 9, 10, 12, 13, 14, 16
2-methylbut-2-en-1-ol (2-methyl-2-buten-1-ol)	11.53	C5H10O	86.13	3, 15, 17, 18
3-hydroxybutan-2-one (acetoin)	11.82	C4H8O2	88.11	1, 2, 3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
(E)-pent-3-en-2-ol (trans-3-penten-2-ol)	11.83	C5H10O	86.13	6
3-methylbutan-1-ol (3-methyl-1-butanol)	12.30	C5H12O	88.15	1, 2, 3, 4, 5, 6, 7, 9, 10,12, 13, 14, 15, 16, 17, 18, 19
2-methylbutan-1-ol (2-methyl-1-butanol)	12.39	C5H12O	88.15	1, 4, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19
(3E)-hepta-1,3,6-triene	12.67	C14H20	188.31	3, 6, 15, 18, 19
(methyldisulfanyl)methane (dimethyl disulfide)	12.70	C2H6S2	94.20	4, 10, 12, 14, 16
2-methylpropanoic acid (isobutyric acid)	12.72	C4H8O2	88.11	4, 5, 7, 9, 10, 12, 13, 14, 16, 17, 18, 19
pentan-1-ol (pentyl alcohol)	12.80	C5H12O	88.15	2, 3, 5, 6, 8, 9, 10, 11, 12, 13, 14, 18, 19
pyridine	12.81	C5H5N	79.10	3, 5, 6, 9, 10, 13, 15, 16, 17, 19
2-sulfanylethanol (2-mercaptoethanol)	13.00	C2H6OS	78.13	10, 12, 13, 14
propyl acetate	13.19	C5H10O2	102.13	4, 7, 8, 10,13, 14
4-methylpentan-2-one (methyl isobutyl ketone)	13.22	C6H12O	100.16	4
butanoic acid (butyric acid)	13.33	C4H8O2	88.11	4, 6, 7, 9, 10, 12, 13, 14, 17
2-methylpropyl acetate (isobutyl acetate)	13.34	C6H12O2	116.16	17, 19
(E)-pent-2-enal (trans-2-pentenal)	13.64	C5H8O	84.12	4, 5, 7, 9, 10, 12, 13, 18
(3E)-1,3-Octadiene	13.68	C8H14	110.20	3,18, 19
hexan-2-one (2-hexanone; butyl methyl ketone)	13.73	C6H12O	100.16	4, 5, 12, 13, 16
2-ethylhexanal (hexanal)	13.99	C8H16O	128.21	1, 4, 5, 7, 8, 9, 10, 12
2-methylbutane (isopentane)	14.10	C5H12	72.15	3
1-propoxypropan-2-ol (1-propoxy-2-propanol)	14.12	C6H14O2	118.17	3
dec-1-ene (1-decene)	14.20	C10H20	140.27	1
2-heptylfuran	14.31	C11H18O	166.26	1, 6
butyl acetate	14.36	C6H12O2	116.16	2, 5, 6, 8, 11, 13, 19
p-cymene	14.50	C10H14	134.22	12
3-methylbutanoic acid (isovaleric acid)	14.63	C5H10O2	102.13	4, 9, 12, 13, 14, 15
2-(methoxymethyl)furan (furfuryl methyl ether)	14.69	C6H8O2	112.13	5, 6, 8, 10, 15, 16, 18
ethyl 2-methylbutyrate	15.03	C7H14O2	130.18	9
1H-pyrrole	15.11	C4H5N	67.09	2, 12, 14
(E)-ethyl but-2-enoate (ethyl butenoate)	15.14	C6H10O2	114.14	17, 19
ethylbenzene	15.26	C8H10	106.17	2, 9, 12, 17
butyl propanoate	15.27	C7H14O2	130.18	17, 19
oct-1-ene	15.38	C8H16	112.21	1
hexan-1-ol (1-hexanol; hexyl alcohol)	15.39	C6H14O	102.17	2, 12, 13, 14, 16, 18
xylene (M-, P-, & O-)	15.44	C8H10	106.17	2, 3, 4, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18
3-methylbutyl acetate (isoamyl acetate)	15.54	C7H14O2	130.18	2, 9, 11, 12, 17, 18, 19
1,4-bis(methylene)cyclohexane (1,4-dimethylencyclohexan)	15.80	C8H12	108.18	3
β-pinene	15.88	C10H16	136.23	2, 11, 13, 14
heptan-2-one (2-heptanone; methyl pentyl ketone)	16.08	C7H14O	114.19	1, 2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16
nonane	16.30	C9H20	128.26	4, 5, 11, 14, 19
styrene	16.52	C8H8	104.15	2, 3, 5, 6, 7, 8, 9, 10, 11, 12, 16, 17, 18, 19
(3E,5E)-octa-1,3,5-triene (octatriene; AC1NSPAH)	16.60	C8H12	108.18	3, 18
oxolan-2-one (γ-Butyrolactone)	16.65	C4H6O2	86.09	12, 13, 14, 15, 16, 17, 18
heptanal (heptyl aldehyde)	16.72	C7H14O	114.19	1, 2, 5, 7, 8, 9, 10, 11, 12, 13, 14, 16

Table 2. Headspace volatile collection of *Trichoderma* isolates (100 ml/min, purge rate, 4 hr, 1 µg Int. Std. by P&T-TD-GC-MS).

Chemical Name (alternate name)	RT (min)	Chemical Formula	MW (g/mol)	*Trichoderma* Isolates
heptanal (heptyl aldehyde)	16.72	C7H14O	114.19	1, 2, 5, 7, 8, 9, 10, 11, 12, 13, 14, 16
2-methylbutanoic acid	16.77	C5H10O2	102.13	4, 5, 7, 9, 13, 17, 18, 19
1-ethyl-2-heptylcyclopropane	16.92	C12H24	168.32	12
3-oxobutan-2-yl acetate (3-acetoxy-2-butanone)	17.20	C6H10O3	130.14	17, 19
3-methylbutyl propanoate (isoamyl propionate)	17.36	C8H16O2	144.21	19
cyclohexanone	17.42	C6H10O	98.14	2, 12, 13
hexanoic acid	17.55	C6H12O2	116.16	3, 4, 5, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19
octan-2-one (2-octanone; methyl hexyl ketone)	17.62	C8H16O	128.21	1, 4, 5, 7, 8, 9, 10, 11, 12
2-pentylfuran	17.62	C9H14O	138.21	1, 2, 7, 9, 10, 12, 13, 14
α-phellandrene	17.75	C10H16	136.23	13
1-(2-Furyl)ethanone (2-acetylfuran)	17.90	C6H6O2	110.11	12, 16, 17, 18, 19
3-carene	18.01	C10H16	136.24	2, 9, 13
5-methylheptan-3-one (5-methyl-3-heptanone)	18.10	C8H16O	128.21	4, 7, 12, 16, 18
octan-3-one (3-octanone; ethyl amyl ketone)	18.14	C8H16O	128.21	3, 5, 6, 9, 12, 13, 14, 15, 16, 17, 18, 19
octan-3-ol (3-octanol)	18.32	C8H18O	130.23	4, 5, 12, 13, 16, 17, 18
(2E,4E)-2,4-Heptadienal (trans-2,4-Heptadienal)	18.32	C7H10O	110.15	18
β-ocimene	18.43	C10H16	136.23	2, 11
2,4-dimethylpentan-3-yl acetate (2,4-dimethyl-3-pentanol acetate)	18.45	C9H18O2	158.24	18
β-phellandrene	18.54	C10H16	136.23	13
2,4,6-trimethyl-decane	18.55	C13H28	184.36	19
6-methylhept-5-en-2-one (sulcatone)	18.59	C8H14O	126.20	1, 12
octanal	18.72	C8H16O	128.21	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
1-methyl-2-methylene-cyclopentane	18.74	C7H12	96.17	17
benzonitrile	18.91	C7H5N	103.12	3, 7, 14
2-ethylhexan-1-ol (ethylhexanol)	19.00	C8H18O	130.23	3, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
ethyl (2E,4E)-hexa-2,4-dienoate (ethyl sorbate)	19.09	C8H12O2	140.18	18
(E)-oct-2-enal (trans-2-Octenal)	19.20	C8H14O	126.20	1
limonene	19.28	C10H16	136.23	1, 2, 3, 4, 5, 6, 7, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
terpinolene	19.35	C10H16	136.23	2, 13, 16
1-ethylcyclohexene	19.53	C8H14	110.20	17, 19
2-phenylacetaldehyde (benzeneacetaldehyde)	19.57	C8H8O	120.15	4, 5, 6, 7, 10, 13, 14, 16, 17
nonan-2-one (2-nonanone; heptyl methyl ketone)	19.70	C9H18O	142.24	2, 4, 5, 7, 8, 9, 10, 11, 12, 13, 14, 16
octan-1-ol (1-octanol; octyl alcohol)	19.75	C8H18O	130.23	1, 16
2-methyl-5-propan-2-ylcyclohexan-1-one (tetrahydrocarvone)	19.94	C10H18O	154.25	3, 6, 17
octan-3-yl acetate (3-octyl acetate; amyl ethyl carbinyl acetate)	20.26	C10H20O2	172.26	12
undecane	20.36	C11H24	156.31	1, 3, 5, 6, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19
nonanal	20.51	C9H18O	142.24	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
phenol	20.67	C6H6O	94.11	2, 3,4, 6, 7, 14, 15, 18
3-Octanyl acetate	20.69	C10H20O2	172.27	4, 16, 17, 18, 19
2-phenylethanol (phenethyl alcohol)	20.93	C8H10O	122.16	11, 12, 13, 16, 18
1-ethenyl-2,4-dimethylbenzene (2,4-dimethylstyrene)	21.12	C10H12	132.20	5, 7, 8,12, 13, 14
(1R,4S)-1-methyl-4-propan-2-ylcyclohex-2-en-1-ol	21.13	C10H18O	154.25	2, 12, 13
2-ethylhexyl acetate	21.22	C10H20O2	172.26	4, 12, 15, 16, 18, 19
2-Isopropyl-5-methyl-6-oxabicyclo[3.1.0]hexane-1-carbaldehyde	21.22	C10H16O2	168.23	17, 19
nonan-1-ol (1-nonanol; nonyl alcohol)	21.30	C9H20O	144.25	4, 8, 10
methyl benzoate	21.32	C8H8O2	136.15	17, 18, 19
(E)-undec-2-en-1-ol (trans-2-undecen-1-ol)	21.87	C11H22O	170.29	9
5-hydroxy-5-methyl-2-propan-2-ylcyclohexan-1-one	21.94	C10H18O2	170.25	2, 12, 13
camphor	22.14	C10H16O	152.23	2, 4, 5, 6, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18
decan-2-one (2-decanone; octyl methyl ketone)	22.21	C10H20O	156.27	2, 4, 5, 7, 8, 9, 10, 12, 13, 14
decanal	22.35	C10H20O	156.27	1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19
(1R,6S)-3-methyl-6-propan-2-ylcyclohex-2-en-1-ol (piperitol)	22.43	C10H18O	154.25	2, 12, 13
methyl salicylate	22.56	C8H8O3	152.15	2, 8, 11
benzoic acid	22.80	C7H6O2	122.12	3, 6, 7, 9, 10, 12, 13, 14, 15, 16, 17, 18, 19
nonanoic acid	23.10	C9H18O2	158.24	3, 4, 5, 6, 7, 9, 10, 12, 13, 16, 17, 18, 19
undecan-2-one (2-undecanone; methyl nonyl ketone)	23.34	C11H22O	170.29	4, 7, 8, 9, 10
5-methyl-1,2,3,4-tetrahydronaphthalene (5-methyltetralin)	23.62	C11H14	146.23	2, 5, 8, 11, 19
2-ethyl-2-propylhexan-1-ol	23.72	C11H24O	172.31	2, 9, 12, 13
β-myrcene	23.72	C10H16	136.23	3

Table 2. Headspace analysis (continued).

Chemical Name (alternate name)	RT (min)	Chemical Formula	MW (g/mol)	*Trichoderma* Isolates
undecanal	23.80	C11H22O	170.29	4, 7, 8, 9, 10, 13, 16
1,3-benzothiazole	23.92	C7H5NS	135.19	13
quinoline	24.16	C9H7N	129.16	2, 9, 13, 17
β-cedrene	24.48	C15H24	204.35	4, 6, 9, 15, 16, 17, 19
aromadendrene	24.60	C15H24	204.35	1
(1E,5E)-1,6-Dichloro-1,5-cyclooctadiene	24.68	C8H10Cl2	177.07	6, 10, 16
α-cedrene	25.08	C15H24	204.35	6, 13, 15, 16, 17, 18, 19
α-farnesene	25.19	C15H24	204.35	1, 2, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 19
β-cubebene	25.20	C15H24	204.35	15, 16, 17, 18
pentadecane	25.20	C15H32	212.41	7, 10, 14
octadecane	25.40	C18H38	254.49	9
α-copaene	25.45	C15H24	204.35	12, 13, 15, 19
β-bisabolene	25.81	C15H24	204.35	12, 13, 15, 16, 17, 18, 19
α-acoradiene	25.89	C15H24	204.35	13
α-bergamotene (2-norpinene)	25.98	C15H24	204.35	2, 4, 5, 7, 8, 9, 11, 12, 13, 14, 15, 16, 19
β-farnesene	26.14	C15H24	204.35	1, 2, 4, 5, 7, 8, 9, 11, 12, 13, 14, 15, 18, 19
β-himachalene	26.20	C15H24	204.35	1, 9, 14
4-Isopropyl-1,6-dimethyl-1,2,3,4,4a,7-hexahydronaphthalene	26.26	C15H24	204.35	6, 15, 16
β-sesquiphellandrene	26.59	C15H24	204.35	7, 9, 13, 19
2,6-ditert-butyl-4-methylphenol (butylated hydroxytoluene)	26.84	C15H24O	220.35	2, 5, 7, 8, 9, 10, 11, 12, 13, 14
isocaryophyllene (β-caryophyllene)	27.03	C15H24	204.35	2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14,15, 16, 18, 19
γ-himachalene	27.05	C15H24	204.35	4, 5, 9
propan-2-yl dodecanoate (isopropyl dodecanoate; isopropyl laurate)	27.15	C15H30O2	242.40	2, 5, 8, 11, 19
β-acoradiene	27.36	C15H24	204.35	3, 4, 6, 7, 9, 13, 15, 16, 17, 18, 19
heptadecane	27.85	C17H36	240.47	2, 4, 5, 8, 9, 10, 11, 12, 14, 19
6-pentylpyran-2-one (6-Pentyl-2H-pyran-2-one)	28.74	C10H14O2	166.22	4, 8, 9, 10, 12
methyl dihydrojasmonate	30.10	C13H22O3	226.31	2, 5, 8, 11
kaur-15-ene	30.50	C20H32	272.47	1
(1E,5E,9E)-1,5,9-Trimethyl-1,5,9-cyclododecatriene	33.33	C15H24	204.35	17, 19

	Trichoderma Isolates
1	*T. atroviride* (JWB)
2	*T. viride* (GJS 04-379)
3	*T. brevicompactum* (CBS 109720)
4	*T. aggressivum* (DAOM 222156)
5	*T. viride* (BBA 70239)
6	*T. harzianum* (CBS 227.95)
7	*T. asperellum* (CBS 433.97)
8	*T. atroviride* (GJS 01-209)
9	*T. asperellum* (GJS 02-65)
10	*T. aggressivum* (IMI 393970)
11	*T. atroviride* (CBS 351.93)
12	*T. virens* (DAOM 167651)
13	*H. koningii* (CBS 989.97)
14	*T. stromaticum* (GJS 00-127)
15	*T. inhamatum* (CBS 273.78)
16	*T. pseudokoningii* (CBS 130756)
17	*T. pseudokoningii* (CBS 480.91)
18	*T. longibrachiatum* (CBS 118642)
19	*T. longibrachiatum* (TR 97)

Table 2. Headspace analysis (continued).

The average concentrations of volatile metabolites in triplicates and the accompanying heatmap are provided in Supplemental Table 1 and Supplemental Figure 1. We completed one-way ANOVA and Tukey's HSD tests ($P < 0.05$). We completed pairwise statistical tests between isolates in same species such as *T. viride* (BBA 70239) and *T. viride* (GJS 04-379). While isolates differed in volatile abundance, the concentrations of individual compounds were not significantly differerent.

4.4 Discussion

Plant growth promotion induced by bacterial VOCs has been demonstrated mostly for strains of *Bacillus* with agricultural significance (Ryu et al., 2003; 2004; Cortest-Barco et al., 2010; Blom et al., 2011). The volatile profile of bacteria or fungi is dependent on multiple parameters such as geography, time, and environmental conditions such as nutrient content, microbial community composition, temperature, humidity, and pH (McNeal and Herbert, 2009; Insam and Seewald, 2010; Polizzi et al., 2012; Lee et al., 2015).

We screened 20 *Trichoderma* isolates comprising of 12 species using a simple plate-within-a-plate method consisting of a large partitioned Petri dish with plant seeds growing on one side with a smaller Petri dish containing sporulating cultures of *Trichoderma*. We demonstrated that 9 isolates significantly improved plant growth in *Arabidopsis* including biomass, plant size, and chlorophyll concentration. *T. atroviride* isolate inhibited plant growth with localized death in leaves and reduction in plant size and biomass. It has been proposed that the production of CO_2 by microorganisms and increase in the CO_2 concentration in Petri plate system can lead to a plant growth promotion (Kai and Piechulla, 2009). In our study, we did not find significant difference in the level of CO_2 by *Trichoderma* and ambient air in the control conditions.

Furthermore, sequestering *Trichoderma* produced CO_2 by absorption in the Petri system did not reduce the growth promotion observed.

Trichoderma isolates emitting plant stimulatory VOCs were geographically diverse and belonged to several clades, among these, the majority of the beneficial VOC producing isolates belong to the Harzianum and Longibrachiatum clades. Several different *Trichoderma* species are used currently as biocontrol agents in commercial agricultural products. *T. harzianum* grows on plant roots and when the fungal hyphae penetrate root epidermis, the fungi induce plant growth and enhance the plant immune system (Harman et al., 2004; Friedl and Druzhinina, 2012; Martinez-Medina et al., 2013). *T. asperellum* (GJS 02-65) is a potential biocontrol agent against root rot pathogens (Mbarga et al., 2012). Pretreatment of plants with *T. asperellum* reduced pathogen infection up to 50%.

The *Arabidopsis* bioassay allowed us to screen volatile-mediated effects. We further measured the plant response using *Solanum lycopersicum* (tomato) seedlings with the growth promoting *Trichoderma* isolate identified in this screen. Tomato response was similar to *Trichoderma* VOC-induced *Arabidopsis* growth promotion, with a significant increase in plant biomass, larger plant size, and increased lateral root development. Continuous exposure to VOCs was similar to previous reports of growth promotion in *Arabidopsis* where sustained exposure led to increases in plant growth (Hung et al., 2013).

Trichoderma species produce many volatile metabolites. For example, *T. harzianum* produces more than 278 volatile compounds, ranging in size from C7 to C30 (Siddiquee et al., 2012). *Trichoderma* volatiles can be self inhibitory, inhibit other fungi,

or induce sexual mating in certain microbial species, leading to the suggestion that the volatile themselves might have applications as biological control agents (Wheatley et al., 1997; Bruce et al., 2000; Aguero et al., 2008; Campos et al., 2010). Several *Trichoderma* species and strains previously have been studied for their volatile production including: *T. atroviride* (Nemcovic et al., 2008; Stoppacher et al, 2010; Polizzi et al., 2011), *T. aureoviride* (Bruce et al., 2000), *T. harzianum* (Fiedler et al., 2001; Siddiquee et al., 2012), *T. pseudokoninggi* (Wheatley et al., 1997), and *T. viride* (Wheatley et al., 1997; Wilkins et al., 2000; Hung et al., 2013).

We performed GC-MS analysis of the volatiles produced by *Trichoderma* isolates and we identified more than 147 compounds. The volatile profiles differed depending on the isolate tested. The production of terpenes, especially sesquiterpenes differed. Eight-carbon volatiles are ubiquitous and characteristics of numerous fungi, accounting for 44 - 97% of the total volatile fraction reported (Combet et al., 2006). The C8 volatile, 1-octen-3-ol, is a common fungal VOCs and has been reported from several *Trichoderma* species (Siddiquee et al., 2012; Mukherjee et al., 2013); however, we did not find this compound in our analysis. However, we found several other related C8 compounds including 3-octanol, octanol, and 3-octanone.

Other common microbial voaltiles previously reported from bacteria and fungi were found in our studies and include 3-methyl-1-butanol, 2-methyl-1-propanol, limonene, β-farnesene, and β-caryophyllene (Pasanen et al., 1997; Fiedler et al., 2001; Korpi et al., 2009; Jelen et al., 2014). Some of these compounds have activity in plants. For example, plants exposed to the volatile phase of 3-methyl-1-butanol, limonene, acetoin, and β-caryophyllene were larger, more robust plants with increased chlorophyll

concentration, while exposure to β-farnesene reduced growth (Ryu et al., 2003; Lee et al., 2015b). Common compound such as butan-2-one detected from multiple isolates produced similar quantities. We also found that 1-hexanol producing isolates did not stimulate or inhibit plant growth. Other research has shown that exposure to 1-hexanol results in inhibition of *Arabidopsis* growth (Splivallo et al., 2007). It is important to note that the biological activities of these individual compounds are also dependent on the concentration and duration of the exposure.

Many *Trichoderma* species produce 6-pentyl-2H-pyran-2-one (6PP), a lactone with a coconut-like odor, and we also showed that 5 *Trichoderma* isolates in this study produced 6PP in high concentrations. However, the presence of 6PP was not unique to all promoting isolates and other isolates in this study did not produce this compound. In addition to its use as flavoring agent, this compound has been studied for bioactivity against other organisms such as *Fusarium* and *Rhizoctonia* (De Souza Ramos et al., 2008; Hanson, 2005; Cooney et al., 2001; Scarselletti et al., 1994). At different concentrations, 6PP promotes or inhibits seedling growth (Harman et al., 2004; Vinale et al., 2008).

In agriculture, *Trichoderma* species have been commercialized as plant growth promoters and for protection against pathogens. Select *Trichoderma* species, such as *T. harzianum* and *T. viride*, important in agriculture have been analyzed for their volatile productions and a large number of compounds have been identified notably including pyrones and sesquiterpenes. There is increasing evidence that some *Trichoderma* volatiles are bio-stimulatory and have the potential to enhance plant growth and development (Vinale et al., 2008; Hung et al., 2013). Since these studies were conducted with different species, exposure methods, and VOC analytical methods, they are not easy

to compare. Here, the effect of 20 *Trichoderma* isolates on plant growth, and a survey of their volatile productions were compared using standardized conditions. To our knowledge 19 out of 20 *Trichoderma* isolates were studied for the first time. Our work suggests that *Trichoderma* volatiles have hitherto underutilized potential as plant growth protectors and/or disease suppressors. We hypothesize that a number of biogenic VOCs could become a powerful tool in agriculture and environmental fields.

REFERENCES

Aarti, T., Meenu, S. (2015). Role of volatile metabolites from *T. citrinoviride* in biocontrol of phytopathogens. International Journal of Research in Chemistry and Environment. 5: 86-95.

Aguero, L.E.M., Alvarado, R., Martinez, A., Dorta, B. (2008). Inhibition of *Aspergillus flavus* growth and aflotoxin B1 production in stored maize grains exposed to volatile compounds of *Trichoderma harzianum* rifai. Interciencia. 33: 219-222.

Bishnoi, N.R., Kumar, R., Bishnoi, K. (2007). Biosorption of Cr (VI) with *Trichoderma viride* immobilized fungal biomass and cell free Ca-alginate beads. Indian Journal of Experimental Biology. 45: 657-664.

Bitas, V., Kim, H.-S., Bennett, J., Kang, S. (2013). Sniffing on microbes: diverse roles of microbial volatile organic compounds in plant health. Molecular Plant-Microbe Interactions Journal. 4: 835-843.

Blom, D., Fabbri, C., Connor, E.C., Schiestl, F.P., Klauser, D.R., Boller, T., Eberl, L., Weisskopf, L. (2011). Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. Environmental Microbiology. 13: 3047-3058.

Bruce, A., Wheatley, R.E., Humphris, S.N., Hackett, C.A., Florence, M.E.J. (2000). Production of volatile organic compounds by *Trichoderma* in media containing different amino acids and their effect on selected wood decay fungi. Holzforschung. 54: 481-486.

Campos, V.P., Pinho, R.S.C. de, Freire, E.S. (2010). Volatiles produced by interacting microorganisms potentially useful for the control of plant pathogens. Ciencia e Agrotecnologia, Lavras. 34: 525-535.

Chakraborty, M.R., Chatterjee, N.C. (2008). Control of *Fusarium* wilt of *Solanum melongena* by *Trichoderma* spp. Biologia Plantarum. 52: 582-586.

Collins, R.P., Halim, A.F. (1972). Characterization of the major aroma constituent of the fungus *Trichoderma viride* (Pers.). Journal of Agricultural and Food Chemistry. 20: 437-738.

Combet, E., Henderson, J., Eastwood, D.C., Burton, K.S. (2006). Eight carbon volatiles in fungi: properties, analysis, and biosynthesis. Mycoscience. 47: 317-326.

Cooney, J.M., Lauren, D.R., Di Menna, M.E. (2001). Impact of competitive fungi on trichothecene production by *Fusarium graminearum*. Journal of Agricultural and Food Chemistry. 49: 522-526.

Cortes-Barco, A.M., Goodwin, P.H., Hsiang, T. (2010). Comparison of induced resistance activated by benzothiadiazole, (2R, 3R)-butanediol and an isoparaffin mixture against anthracnose of *Nicotiana benthamiana*. Plant Pathology. 59: 643-653.

Dennis, C., Webster, J. (1971). Antagonistic properties of species-groups of *Trichoderma*: II. Production of volatile antibiotics. Transactions of the British Mycologial Society. 57: 41-48.

Friedl, M.A., Druzhinina, I.S. (2012). Taxon-specific metagenomics of *Trichoderma* reveals a narrow community of opportunistic species that regulate each other's development. Microbiology. 158: 69-83.

Fiedler, K., Schutz, E., Geh, S. (2001). Detection of microbial volatile organic compounds (MVOCs) produced by moulds on various materials. International Journal of Hygene Environmental Health. 204: 111-121.

Hanson, J.R. (2005). The chemistry of the bio-control agent, *Trichoderma harzianum*. Science Progress. 88: 237-248.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M. (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. Nature Reviews Microbiology. 2: 43-56.

Hung, R., Lee, S., Bennett, J.W. (2013). *Arabidopsis thaliana* as a model system for testing the effects of *Trichoderma* volatile organic compounds. Fungal Ecology. 6: 19-26.

Hung, R., Lee, S., Bennett, J.W. (2015). Fungal volatile organic compounds and their role in ecosystems. Applied Microbiology and Biotechnology. 99: 3395-3405.

Insam, H., Seewald, S.A. (2010). Volatile organic compounds (VOCs) in soils. Biology and Fertility of Soils. 46: 199-213.

Jelen, H., Blaszczyk, L., Jerzy, C., Rogowicz, K., Strakowska, J. (2014). Formation of 6-n-pentyl-2H-pyran-2-one (6-PAP) and other volatiles by different *Trichoderma* species. Mycological Progress. 13: 589-600.

Kai, M., Piechulla, B. (2009). Plant growth promotion due to rhizobacterial volatiles - an effect of CO_2? FEBS Letters. 583: 3473-3477.

Kanchiswamy, C.N., Malnoy, M., Maffei, M.E. (2015). Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Frontiers in Plant Science. 6: 151-174.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J. (2007). Volatile 1-octen-3-ol induces a defensive response in *Arabidopsis thaliana*. Journal of Plant Pathology. 73: 35-37.

Korpi, A., Jarnberg, J., Pasanen, A.L. (2009). Microbial volatile organic compounds. Critical Reviews in Toxicology. 39: 139-193.

Kubicek, C.P. (2013). Systems biological approaches towards understanding cellulase production by *Trichoderma reesei.* Journal of Biotechnology. 163: 133-142.

Kudalkar, P., Strobel, G., Hasan, S.R.U., Geary, G., Sears, J. (2012). *Muscodor sutura* a novel endophytic fungus with volatile antibiotic activities. Mycoscience. 53: 319-325.

Lee, S., Hung, R., Yap, M., Bennett, J.W. (2015). Age matters: the effects of volatile organic compounds emitted by *Trichoderma atroviride* on plant growth. Archives of Microbiology. 197: 723-727.

Martinez-Medina, A., Fernandez, I., Sanchez-Guzman, M.J., Jung, S.C., Pascual, J.A., Pozo, M.J. (2013). Deciphering the hormonal signaling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. Frontiers in Plant Science. 4: 206-218.

Mathivanan, N., Prabavathy, V.R., Vijayanandraj, V.R. (2008). The effect of fungal secondary metabolites on bacterial and fungal pathogens. In: Karlovsky, P. (Ed.) Secondary metabolites in soil ecology. Springer-Verlag. Pgs. 129-140.

Mbarga, J.W., Martijin Ten Hoopen, G., Kuate, J., Adiobo, A., Ngonkeu, M.E.L., Ambang, Z., Akoa, A., Tondje, P.R., Begoude, B.A.D. (2012). *Trichoderma asperellum*: a potential biocontrol agent for *Pythium myriotylum* causal agent of cocoyam (*Xanthosoma sagittifolium*) root rot disease in Cameroon. Crop Protection. 36: 18-22.

Mitchell, A.M., Strobel, G.A., Moore, E., Robison, R., Sears, J. (2010). Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus. Microbiology. 156: 270-277.

Morales-Barrera, L., Cristiani-Urbina, E. (2008). Hexavlent chromium removal by a *Trichoderma inhamatum* fungal strain isolated from tannery effluent. Water, Air, Soil Pollut. 187: 327-336.

Morath, S.U., Hung, R., Bennett, J.W. (2012). Fungal volatile organic compounds: a review with emphasis on their biotechnological potential. Fungal Biology Reviews. 26: 73-83.

Mukherjee, P.K., Horwitz, B.A., Kenerley, C.M. (2012). Secondary metabolism in *Trichoderma* - a genomic perspective. Microbiology. 158: 35-45.

Mukherjee, P.K., Horwitz, B.A., Singh, U.S., Mukherjee, M., Schmoll, M. (Ed.) (2013). *Trichoderma* Biology and Applications. CAB International. Pgs. 1-9.

Nemcovic, M., Jakubikova, L., Viden, I., Farkas, V. (2008). Induction of conidiation by endogenous volatile compounds in *Trichoderma* spp. FEMS Microbiology Letters. 284: 231-236.

Pasanen, P., Korpi, A., Kalliokosi, P., Pasanen, A.L. (1997). Growth and volatile metabolite production of *Aspergillus versicolor* in house dust. Environment International. 23: 425-432.

Penuelas, J., Asensio, D., Thol, D., Wenke, K., Rosenkranz, M., Piechulla, B., Schnitzler, J.P. (2014). Biogenic volatile emissions from the soil. Plant, Cell and Environment. 37: 1866-1891.

Polizzi, V., Adams, A., Picco, A.M., Adriaens, E., Lenoir, J., Peteghem, C.V., Saegar, S.D., Kimpe, N.D. (2011). Influence of environmental conditions on production of volatiles by *Trichoderma atroviride* in relation with sick building syndrome. Building and Environment. 46: 945-954.

De Souza Ramos, A., Fiaux, S.B., Leite, S.G.F. (2008). Production of 6-pentyl-α-pyrone by *Trichoderma harzianum* in solid-state fermentation. Brazilian Journal of Microbiology. 39: 712-717.

Raza, W., Faheem, M., Yousaf, S., Rajer, F.U., Yameen, M. (2013). Volatile and non-volatile antifungal compounds produced by *Trichoderma harzianum* SQR-T-037 suppressed the growth of *Fusarium oxysporum* f. sp. niveum. Science Letters. 1: 21-24.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wie, H.-X., Pare, P.W., Kloepper, J.W. (2003). Bacterial volatiles promote growth of *Arabidopsis*. Proceedings of the National Academy of Sciences. 100: 4927-4932.

Ryu, C.M., Farag, M.A., Hu, C.H., Reddy, M.S., Kloepeer, J.W., Pare, P.W. (2004). Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiology. 134: 1017-1026.

Scarselletti, R., Faul, J.L. (1994). In vitro activity of 6-pentyl-α-pyrone, a metabolite of *Trichoderma harzianum*, in the inhibition of *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. lycopersici. Mycological Research. 98: 1207-1209.

Schuster, A., Schmoll, M. (2010). Biology and biotechnology of *Trichoderma*. Applied Microbiology and Biotechnology. 87: 787-799.

Siddiquee, S., Cheong, B.E., Taslima, K., Kausar, H., Hasan, M. (2012). Separation and identification of volatile compounds from liquid cultures of *Trichoderma harzianum* by GC-MS using three different capillary columns. Journal of Chromatographic Science. 50: 358-367.

Splivallo, R., Novero, M., Bertea, C.M., Bossi, S., Bonfante, P. (2007). Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist. 175: 417-424.

Stoppacher, N., Kluger, B., Zeilinger, S., Krska, R., Schuhmacher, R. (2010). Identification and profiling of volatile metabolites of the biocontrol fungus *Trichoderma atroviride* by HS-SPME-GC-MS. Journal of Microbiological Methods. 81: 187-193.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C. (2001). Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Mirobiology. 147: 2943-2950.

Tripathi, P., Singh, P.C., Mishra, A., Chauhan, P.C., Dwivedi, S., Bais, R.T., Tripathi, R.D. (2013). *Trichoderma*: a potential bioremediatior for environmental clean up. Clean Technologies and Environmental Policy. 15: 541-550.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L., Lorito, M. (2008). *Trichoderma*-plant pathogens interactions. Soil Biology and Biochemistry. 40: 1-10.

Wheatley, R., Hackett, C., Bruce, A., Kundzewicz, A. (1997). Effect of substrate composition on production of volatile organic compounds from *Trichoderma* spp. inhibitory to wood decay fungi. International Biodeterioration & Biodegradation. 39: 199-205.

Whipps, J.M., Lumsden, R.D. (Ed.) (1989). Biotechnology of Fungi for Improving Plant Growth. Cambridge University Press.

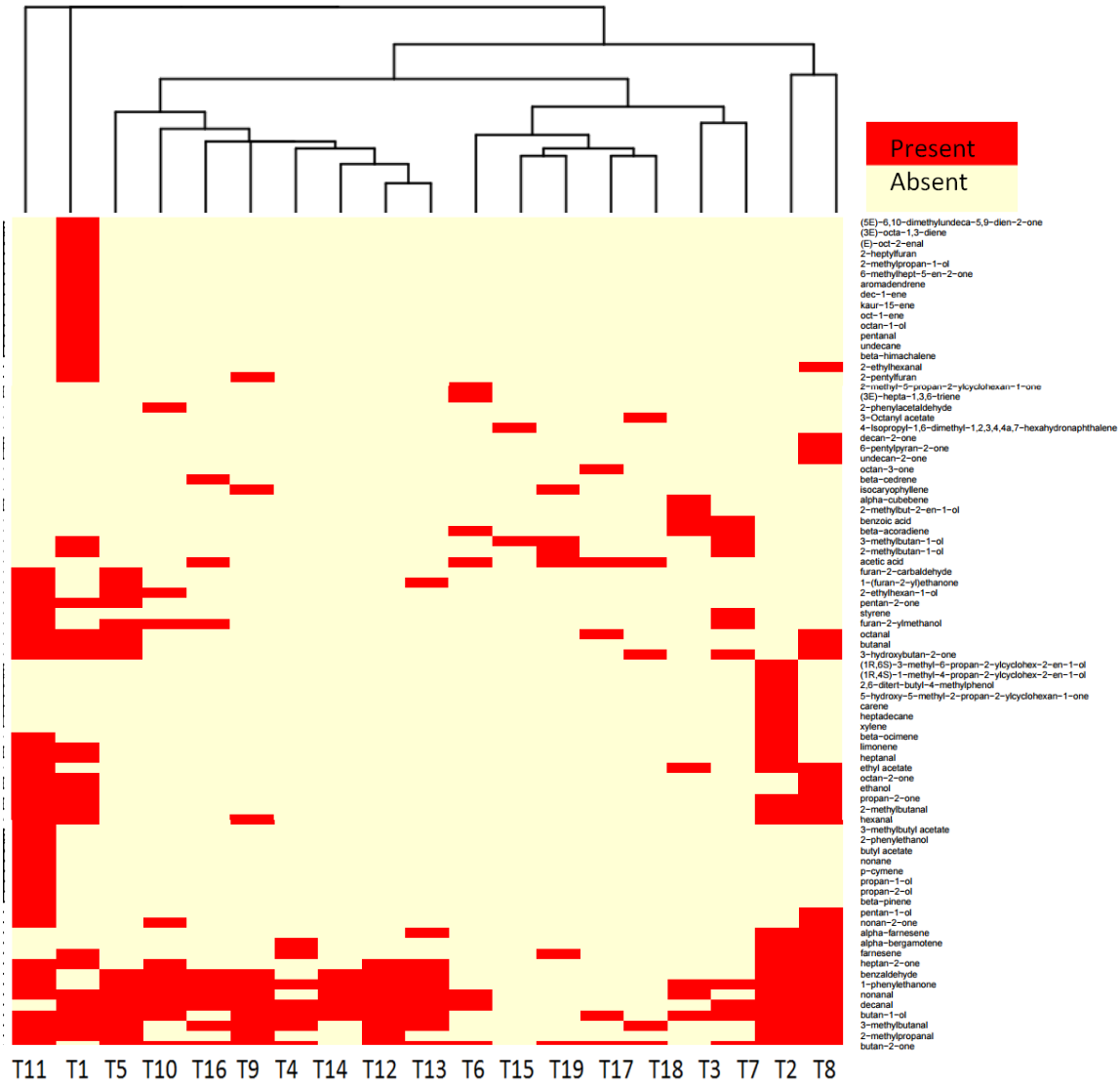
Wilkins, K., Larsen, K., Simkus, M. (2000). Volatile metabolites from mold growth on building materials and synthetic media. Chemosphere. 41: 437-446.

Zhang, F., Yang, X., Ran, W., Shen, Q. (2014). *Fusarium oxysporum* induces the production of proteins and volatile organic compounds by *Trichoderma harzianum* T-E5. FEMS Microbiology Letters. 359: 116-123.

Supplemental Table 1. The production of volatile organic compounds produced by *Trichoderma* isolates identified using headspace GC-MS. The average concentration of compounds found in triplicates purged for 4hours. (—) denotes absence of compound.

Compound	*T. atroviride* (JWB)	*T. viride* (GJS 04-379)	*T. brevicompactum* (CBS 109720)	*T. aggressivum* (DAOM 222156)	*T. viride* (BBA 70239)	T. hatzianum (CBS 227.95)	*T. asperellum* (CBS 433.97)	*T. atroviride* (GJS 01-209)	*T. asperellum* (GJS 02-65)	*T. aggressium* (IMI 393970)	*T. atroviride* (CBS 351.93)	*T. virens* (DAOM 167651)	*H. koningii* (CBS 989.97)	*T. stromaticum* (GJS 00-127)	*T. inhamatum* (CBS 273.78)	*T. pseudokoningii* (CBS 130756)	*T. pseudokoningii* (CBS 480.91)	*T. longibrachiatum* (CBS 118642)	*T. longibrachiatum* (TR 97)
(1R,4S)-1-methyl-4-propan-2-ylcyclohex-2-en-1-ol	—	19.52	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
(1R,6S)-3-methyl-6-propan-2-ylcyclohex-2-en-1-ol	—	263.63	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
(3E)-hepta-1,3,6-triene	—	—	—	—	—	43.59	—	—	—	—	—	—	—	—	—	—	—	—	—
(3E)-octa-1,3-diene	6.06	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
(5E)-6,10-dimethylundeca-5,9-dien-2-one	2.64	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
(E)-oct-2-enal	3.05	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1-(2-Furyl)ethanone (2-acetylfuran)	—	—	—	—	62.02	—	—	—	—	—	10.46	—	33.60	—	—	—	—	—	—
1-phenylethanone	—	—	21.33	—	—	—	8.53	—	—	—	—	—	—	—	—	137.71	—	—	—
2,6-ditert-butyl-4-methylphenol	—	53.38	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2-ethylhexan-1-ol	—	—	—	—	18.30	—	—	—	—	14.92	4.46	—	—	—	—	—	—	—	—
2-ethylhexanal	1.23	—	—	—	—	—	—	50.34	—	—	—	—	—	—	—	—	—	—	—
2-heptylfuran	1.30	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2-methyl-5-propan-2-ylcyclohexan-1-one	—	—	—	—	—	285.13	—	—	—	—	—	—	—	—	—	—	—	—	—
2-methylbut-2-en-1-ol	—	—	50.52	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2-methylbutan-1-ol	49.85	—	—	—	—	—	604.71	—	—	—	—	—	—	—	—	—	—	—	1071.27
2-methylbutanal	21.88	110.17	—	—	—	—	—	48.00	—	—	61.67	—	—	—	—	—	—	—	—
2-methylpropan-1-ol	99.28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2-methylpropanal	13.33	28.93	—	—	36.54	—	—	62.98	87.48	—	19.27	20.51	—	—	—	—	—	—	—
2-pentylfuran	8.51	—	—	—	—	—	—	—	25.56	—	—	—	—	—	—	—	—	—	—
2-phenylacetaldehyde	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2-phenylethanol	—	—	—	—	—	—	—	—	—	—	8.21	—	—	—	—	—	—	—	—
3-hydroxybutan-2-one	1.97	—	—	—	22.76	—	33.02	9.27	—	—	6.17	—	—	—	—	—	—	999.73	—
3-methylbutan-1-ol	85.97	—	—	—	—	—	885.30	—	—	—	—	—	—	—	152.47	—	—	—	918.03
3-methylbutanal	—	—	—	—	—	—	—	—	—	—	2.43	—	38.96	—	—	—	—	—	—
3-methylbutyl acetate	—	—	—	—	—	—	—	—	—	—	3.70	—	—	—	—	—	—	—	—
3-Octanyl acetate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	274.77	—
4-Isopropyl-1,6-dimethyl-1,2,3,4,4a,7-hexahydronaphthalene	—	—	—	—	—	—	—	—	—	—	—	—	—	—	152.32	—	—	—	—
5-hydroxy-5-methyl-2-propan-2-ylcyclohexan-1-one	—	57.65	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6-methylhept-5-en-2-one	5.66	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6-pentylpyran-2-one	—	—	—	—	—	—	—	7559.45	—	—	—	—	—	—	—	—	—	—	—
acetic acid	—	—	—	—	—	407.51	—	—	—	—	—	—	—	—	—	386.95	602.42	959.15	1071.60
aromadendrene	6.34	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
benzoic acid	—	—	27.75	—	—	—	95.92	—	—	—	—	—	—	—	—	—	—	—	—
butan-2-one	—	75.27	—	27.71	48.50	10.17	21.02	33.28	45.79	13.86	57.58	44.54	39.29	—	—	50.47	110.32	48.79	72.46
butanal	2.74	—	—	—	11.90	—	—	3.78	—	—	46.15	—	—	—	—	—	—	—	—
butyl acetate	—	—	—	—	—	—	—	—	—	—	3.18	—	—	—	—	—	—	—	—
3-carene	—	78.06	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
dec-1-ene	54.65	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
decan-2-one	—	—	—	—	—	—	—	6.65	—	—	—	—	—	—	—	—	—	—	—
decanal	—	—	—	—	—	10.06	—	—	—	—	—	—	—	—	—	—	—	—	—
ethanol	6.64	—	—	—	—	—	—	33.69	—	—	26.63	—	—	—	—	—	—	—	—
ethyl acetate	—	76.99	8.32	—	—	—	—	13.93	—	—	39.52	—	—	—	—	—	—	—	—
heptadecane	—	51.28	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
heptan-2-one	2.06	5.77	—	—	—	—	—	155.70	—	61.18	0.59	9.62	19.56	—	—	—	—	—	—
heptanal	7.84	7.41	—	—	—	—	—	—	—	—	3.34	—	—	—	—	—	—	—	—
isocaryophyllene	—	—	—	—	—	—	—	—	53.47	—	—	—	—	—	—	—	—	—	1028.42
kaur-15-ene	14.85	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
limonene	3.04	121.38	—	—	—	—	—	—	—	—	6.00	—	—	—	—	—	—	—	—
nonan-2-one	—	—	—	—	—	—	—	13.28	—	16.34	7.42	—	—	—	—	—	—	—	—
nonanal	—	—	—	—	—	—	—	—	5.95	—	—	—	—	—	—	—	—	—	—
nonane	—	—	—	—	—	—	—	—	—	—	2.67	—	—	—	—	—	—	—	—
oct-1-ene	1.90	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
octan-1-ol	2.56	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
octan-2-one	1.43	—	—	—	—	—	—	4.76	—	—	3.72	—	—	—	—	—	—	—	—
octan-3-one	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	129.92	—	—
octanal	7.42	—	—	—	8.64	—	—	11.70	—	—	7.64	—	—	—	—	—	23.99	—	—
p-cymene	—	—	—	—	—	—	—	—	—	—	1.14	—	—	—	—	—	—	—	—
pentan-1-ol	—	—	—	—	—	—	—	453.35	—	—	609.45	—	—	—	—	—	—	—	—
pentanal	3.91	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
propan-1-ol	—	—	—	—	—	—	—	—	—	—	4.07	—	—	—	—	—	—	—	—
propan-2-ol	—	—	—	—	—	—	—	—	—	—	0.89	—	—	—	—	—	—	—	—
propan-2-one	7.51	225.56	—	—	—	—	—	162.45	—	—	78.92	—	—	—	—	—	—	—	—
styrene	—	—	—	—	—	—	78.88	—	—	—	7.48	—	—	—	—	—	—	—	—
undecan-2-one	—	—	—	—	—	—	—	26.97	—	—	—	—	—	—	—	—	—	—	—
undecane	14.43	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
xylene	—	5.63	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
α-bergamotene	—	160.62	—	183.86	—	—	—	63.19	—	—	—	—	—	—	—	—	—	—	—
β-cubebene	—	—	117.65	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
α-farnesene	—	56.78	—	—	—	—	—	62.87	—	—	—	—	77.57	—	—	—	—	—	—
β-acoradiene	—	—	64.23	—	—	355.38	204.17	—	—	—	—	—	—	—	—	—	—	—	—
β-cedrene	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	726.98	—	—	
β-farnesene	3.70	17.62	—	668.55	—	—	—	23.03	—	—	—	—	—	—	—	—	—	—	9923.40
β-himachalene	40.57	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
β-ocimene	—	1014.69	—	—	—	—	—	—	—	—	14.27	—	—	—	—	—	—	—	—
β-pinene	—	—	—	—	—	—	—	—	—	—	1.09	—	—	—	—	—	—	—	—

Supplemental Figure 1. Heatmap of *Trichoderma* VOCs identified through headspace analysis (in triplicates).



	Trichoderma Isolates		*Trichoderma* Isolates
1	*T. atroviride* (JWB)	11	*T. atroviride* (CBS 351.93)
2	*T. viride* (GJS 04-379)	12	*T. virens* (DAOM 167651)
3	*T. brevicompactum* (CBS 109720)	13	*H. koningii* (CBS 989.97)
4	*T. aggressivum* (DAOM 222156)	14	*T. stromaticum* (GJS 00-127)
5	*T. viride* (BBA 70239)	15	*T. inhamatum* (CBS 273.78)
6	*T. harzianum* (CBS 227.95)	16	*T. pseudokoningii* (CBS 130756)
7	*T. asperellum* (CBS 433.97)	17	*T. pseudokoningii* (CBS 480.91)
8	*T. atroviride* (GJS 01-209)	18	*T. longibrachiatum* (CBS 118642)
9	*T. asperellum* (GJS 02-65)	19	*T. longibrachiatum* (TR 97)
10	*T. aggressivum* (IMI 393970)		

CHAPTER 5.

Effects of Fungal Volatile Organic Compounds on *Arabidopsis thaliana* growth and gene expression

The author is currently preparing a manuscript with results from this chapter which will be submitted for publication.

5.1 Introduction

The production and emission of volatile organic compounds (VOCs) are an important area of research in atmospheric and terrestrial ecosystem studies. They are characterized by low molecular mass, high vapor pressure (>0.01 k Pa), low boiling point and polarity, and readily vaporize and diffuse through the environment (Insam and Seewald, 2010; Penuelas et al., 2014). Volatiles are a portion of the metabolites produced by organisms and their properties enable them to mediate biological functions. There exists a large body of literature investigating volatiles as semio- or info-chemicals for insects and microorganisms and as indicators of microbial contamination in food, food products, and indoor environments. Volatile production is dynamic and is directly affected by an organism's genetic background as well as environmental factors including community composition, substrate, temperature, moisture level, and pH (Sunesson et al., 1995; Claeson et al., 2002; Matysik et al., 2009).

The potential use of microbial VOCs in agriculture includes control of contamination in food products and control of pathogens in plants. Several microbial isolates have been identified for their volatile-mediated fungistatic and antimicrobial activities (Strobel et al., 2001; Chuankun et al., 2004; Vespermann et al., 2007; Mitchell et al., 2010; Kudlkar et al., 2012). In addition, few compounds induce defense responses in plants. For example, exposure to 6-n-pentyl-2H-pyran-2-one stimulates seedling growth while reducing disease symptoms to *Botrytis* and *Leptosphaeria* by inducing an over-expression of pathogenesis-related (PR-1) gene (Vinale et al., 2008). Exposure to 1-octen-3-ol enhanced resistance to *Botrytis* by activating defense genes turned on by ethylene and jasmonic acid signaling (Kishimoto et al., 2007).

Plant growth-promoting rhizobacteria (PGPR), *Bacillus subtilis* and *B. amyloliquefaciens,* improve plant growth by emitting VOCs (Ryu et al., 2003). Moreover, these bacteria produced 2,3-butanediol and direct application of this volatile enhanced plant growth similarly to the blends of *Bacillus* VOCs. Similarly, *Arabidopsis* exposed to blends of *Trichoderma* VOCs were larger in size with increased fresh weight, chlorophyll, and root growth (Hung et al., 2013).

More recently, our lab studied several *Trichoderma* species for their volatile production and screened for volatile-mediated plant growth using *Arabidopsis thaliana* (Lee et al., *In Prep.*). In addition, *Solanum lycopersicum* seedlings exposed to blends of VOCs emitted by growth promoting *Trichoderma* isolate were larger with increased fresh shoot and root weights and total chlorophyll content. More than 147 compounds were identified and comprised of a diverse family of compounds including alcohols, ketones, esters, alkenes, terpenes and their derivatives. Volatile-mediated growth promotion was dependent on the age of the fungal culture, duration of the exposure, and was isolate-specific (Lee et al., 2015a; 2015b).

While the bioactivity of VOCs emitted by microorganisms is evident, little is known about plant response to volatiles emitted by fungi. In literature, both individual and blended VOCs appear to be important in an organism's ability to detect and modify responses. The aim of our study was to evaluate the effects of individual *Trichoderma* VOCs identified in the previous gas chromatography-mass spectrometry (GC-MS) analysis. Twenty six standard compounds were purchased and tested on seed germination, seedling development, and vegetative growth. We identified fungal volatiles that improve *Arabidopsis* growth similar to blends of *Trichoderma* VOCs.

Seven compounds were further tested at a physiological concentration (10 ng) that was determined through GC-MS analysis. Finally, we provide the first global gene expression data for *Arabidopsis* exposed to 1-decene, the single VOC that induced the most pronounced plant growth promotion. Here we present a screening of plant genes and introduce potential candidates for volatile-mediated responses in plants.

5.2 Materials and methods

Plant preparation

Arabidopsis thaliana seeds (ecotype Columbia-7) were surface-sterilized in a 95% ethanol and 20% bleach solution. Surface-sterilized seeds were sown onto a 60 x 15 mm Petri dish containing Murashige and Skoog (MS) medium with vitamins, 3% sucrose, and 0.03% phytagel (pH 5.7) (Phytotechnology Laboratories, KS). Seeds were stratified at 4 °C for three days prior to start of volatile-exposure assays. For the germination assay, 50 seeds were sown onto MS medium and then exposed to standard compounds. For the vegetative exposure assay, five seeds were sown onto MS medium and grown in a growth chamber at 23 $\pm$ 1 ˚C with a 16-hour photoperiod for 14 days prior to the volatile experiment.

Fungal volatile organic compound treatment

Compounds identified through prior GC-MS analysis of *Trichoderma* samples were selected for further study. Twenty six chemical standards were purchased from Sigma-Aldrich Co. (Table 1). Seeds and vegetative plants were exposed to standard compounds as described previously with some modifications (Lee et al., 2014). *Arabidopsis* seeds or 14-day-old vegetative plants were exposed to 0.5 µg of individual

compound. Seven compounds were further tested at 10 ng concentration on vegetative plants.

For the germination assay, a Petri dish containing 50 surface sterilized seeds was placed into a one liter glass tissue culture jar. An appropriate aliquot of each compound was added, volatilized, and the jar was sealed with a translucent polypropylene screw cap. The seeds were exposed to the compound for 72 hours in a growth chamber at 23 $\pm$ 1 ˚C with a 16-hour photoperiod. The control seeds were exposed to the same conditions without the addition of VOCs. At the end of the exposure, the seeds were removed and examined visually using light microscopy. The seeds were scored into three categories, no germination, visual germination (presence of radical), and seedling formation (presence of radical, hypocotyls, and cotyledons).

For vegetative plants, five plants were grown in a growth chamber at 23 $\pm$ 1 ˚C with a 16-hour photoperiod for 14 days following stratification. A Petri plate containing 14-day-old plants was placed into a glass tissue culture jar and an aliquot of the compound of interest was added, volatilized, and the jar sealed and placed in the growth chamber for 72 hr. The plants were removed from experimental conditions, observed for morphological features, individual plants weighed, and total chlorophyll content obtained.

Total chlorophyll content of plants exposed to fungal VOCs was determined by submerging the shoot overnight in 1 ml of 80% acetone in the dark at 4 °C. The total chlorophyll concentration (chlorophyll a and b) was calculated from the equation $[(8.02)(A663) + (20.2)(A645)]V/1000\ W$, where V is volume and W is plant fresh weight. The chlorophyll data were expressed in relation to the fresh weight of the plant shoot.

Three replicates were used per treatment condition, and the experiments were repeated three times. Quantitative results were expressed as standard error of the mean and analyzed using R Statistical Software (version 3.2.1, World Famous Astronaut). One-way analysis of variance (ANOVA) between groups was performed for plant exposure quantitative data.

RNA isolation and sequencing

Fourteen-day-old *Arabidopsis* plants treated with 0.5 µg of 1-decene for 72 h were used for RNA extraction. Six biological replicates were sequenced for treatment to 1-decene and six biological replicates for control. At least four plants were pooled for each biological replicate. Total RNA was obtained from the shoots of *Arabidopsis* plants using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The integrity and purity of the RNA was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Germany).

A TruSeq RNA Sample Preparation kit (Illumina) was used to construct cDNA libraries according to the manufacturer's instructions. Briefly, 4 µg of total RNA was used for polyA selections with RNA purification beads. AMPure XP beads (Beckman Coulter) were used to purify the cDNA and the quality check of the library was performed using the Agilent 2200 TapeStation. Libraries were quantified by the KAPA SYBR FAST qPCR kit (Kapa Biosystems) and pooled at 4 nM concentration. Libraries were denatured with 0.2 N NaOH and sequenced on the Illumina NextSeq 500 Sequencing platform.

Data analysis

The sequencing quality check was performed on the reads using the FastQC software. Since the reads had a high quality score, no trimming was done while mapping the reads. The level of duplication in the sequencing data was assessed and duplicate reads were removed using Picard tools. Full-length reads were mapped onto the *Arabidopsis* genome annotation (TAIR 10) using Bowtie 2 (version 2.2.5). A BAM file for each sample was created by mapping to the genome. The number of reads mapped to every annotated gene in all samples was measured by running BEDTools software package on each BAM file. These read counts were then normalized across samples by calculating the values of reads per kilobase transcript per million (RPKM). The RPKM values provide a normalized measure of read density which allows transcript levels to be compared between samples. Differentially expressed transcripts were identified using R with the DESeq package using a false discovery rate (FDR) < 0.1 as the cut-off for significance. The matrix of read counts for each transcript (41,653) in all 12 samples was used as input for DESeq analysis. The DESeq analysis identifies trends between contexts after correcting for dispersion and the p-value. Fold change between two contexts is used to filter context-specific signatures. The analysis identified genes showing a p-value of less than 0.05 and a fold change greater than 2. Gene Ontology (GO) based enrichment analysis was performed using AgriGO to determine the biological function of differentially expressed genes between control and volatile-treated plants.

5.3 Results

The effects of individual compounds on seed germination and seedling development

Arabidopsis seeds were treated to individual standard compounds identified previously from *Trichoderma* isolates (Table 1). Seeds were treated to each compound at 0.5 µg for 72 hours then germination and seedling development rate was scored visually (Fig. 1). Seeds unaffected by the compound exposure underwent the normal germination process and developed into seedlings comparable to controls. Compounds such as 1-octen-3-ol caused a complete inhibition of seed germination or arrest after radical protrusion (Fig. 1A and Fig. 1B).

Compound Name	Molecular Formula	Structure	Molecular Weight	Family	Odor Description	Effects on Plant Seeds	Effects on Vegetative Plants
butanal	C4H8O		72.11	Aldehyde	Pungent cocoa, musty	Inhibitory	No Effect
1-butanol	C4H10O		74.12	Alcohol	Banana-like, alcoholic, fusel	No Effect	No Effect
2-methyl-1-propanol	C4H10O		74.12	Alcohol	Sweet, musty	No Effect	Growth Promotion
2-methylbutanal	C5H10O		86.13	Aldehyde	Coffee, nutty, musty	Inhibitory	No Effect
3-methylbutanal	C5H10O		86.13	Aldehyde	Apple-like	Inhibitory	No Effect
2-pentanone	C5H10O		86.13	Ketone	Sweet, fruity, banana woody	Inhibitory	No Effect
1-pentanol	C5H12O		88.15	Alcohol	Pungent, bready, solvent-like	Inhibitory	Inhibitory
2-methyl-1-butanol	C5H12O		88.15	Amyl Alcohol	Roasted, wine, onion, fruity	Inhibitory	Growth Promotion
3-methyl-1-butanol	C5H12O		88.15	Amyl Alcohol	Whiskey, fruity banana	Inhibitory	Growth Promotion
2-heptanone	C7H14O		114.19	Ketone	Banana-like, fruity	Inhibitory	Growth Promotion
1-octen-3-one	C8H14O		126.20	Ketone	Metallic, Mushroom-like	Inhibitory	Inhibitory
2-octenal	C8H14O		126.20	Aldehyde	Nutty, Cucumber-like	Inhibitory	Inhibitory
1-octene	C8H16		112.21	Alkene	Gasoline	Inhibitory	No Effect
1-octen-3-ol	C8H16O		128.21	Alcohol	Mushroom	Inhibitory	Inhibitory
3-octanone	C8H16O		128.21	Ketone	Fresh herbal, sweet mushroom	Inhibitory	No Effect
octanoic acid	C8H16O2		144.21	Ester	Faint, rancid, fruity-acid	No Effect	Growth Promotion
1-octanol	C8H18O		130.23	Alcohol	Waxy, orange-rose, mushroom	Inhibitory	No Effect
nonanal	C9H18O		142.24	Aldehyde	Orange-rose	Inhibitory	Inhibitory
6-amyl-α-pyrone	C10H14O2		166.22	Monoterpene	Coconut	No Effect	No Effect
(S)-limonene	C10H16		136.23	Monoterpene	Pine, herbal, peppery	Inhibitory	Growth Promotion
(D)-limonene	C10H16		136.23	Monoterpene	Citrus, orange, fresh sweet	No Effect	Growth Promotion
β-ocimene	C10H16		136.23	Monoterpene	Green, sweet herbal	No Effect	Inhibitory
1-decene	C10H20		140.27	Alkene	Pleasant	No Effect	Growth Promotion
2-heptylfuran	C11H18O		166.26	Heteroaromatic	Nutty, coffee-like	No Effect	Growth Promotion
β-caryophyllene	C15H24		204.35	Sesquiterpene	Woody, spicy	No Effect	No Effect
farnesene	C15H24		204.35	Sesquiterpene	Green apple	No Effect	No Effect

Table 1. Standard fungal volatile organic compounds used in plant bioassays.

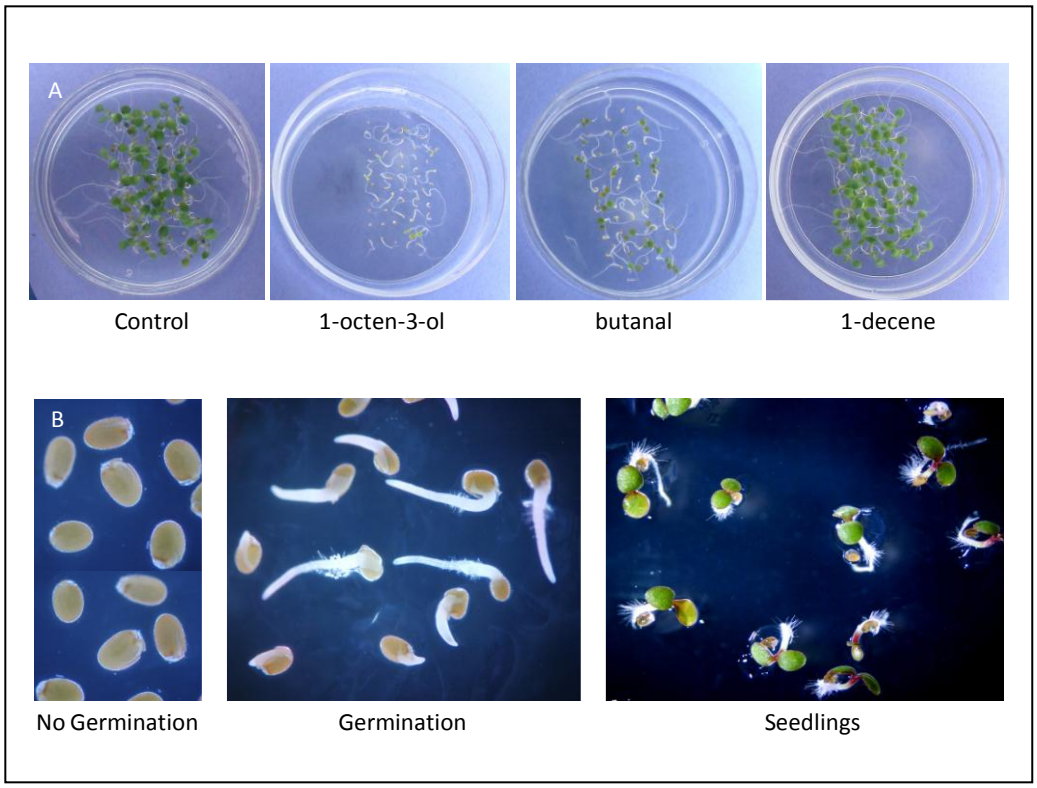


Figure 1. Germination assay of *Arabidopsis thaliana* seeds exposed to individual compounds at 0.5 µl for 72 hours. (A) Visual observation at the end of exposure. (B) Seed germination stages.

In the control, an average of 92 $\pm$ 5.5% of seeds germinated and developed into seedlings (Fig. 2). Fifteen out of 26 compounds tested affected plant seed germination or seedling development negatively ($P < 0.001$). Compounds 1-octen-3-one, 1-octene, and 2-octenal were the most inhibitory leading to 98.6 $\pm$ 0.3%, 79 $\pm$ 2.4%, and 64.7 $\pm$ 8% inhibition of seed germination respectively. Several compounds, 2-methylbutanal, 3-methylbutanal, 2-heptanone, and 3-octanone, lead many seeds (41.3 to 53.9%) to arrest after radical protrusion with no indication of hypocotyl elongation. Exposure to butanal, 1-octanol, and nonanal allowed most seeds to undergo germination; however, approximately 40 - 47% of the germinated seeds developed into seedlings. All seeds

once removed from the exposure conditions resumed the germination process and developed into seedlings at rate comparable to controls (data not shown).

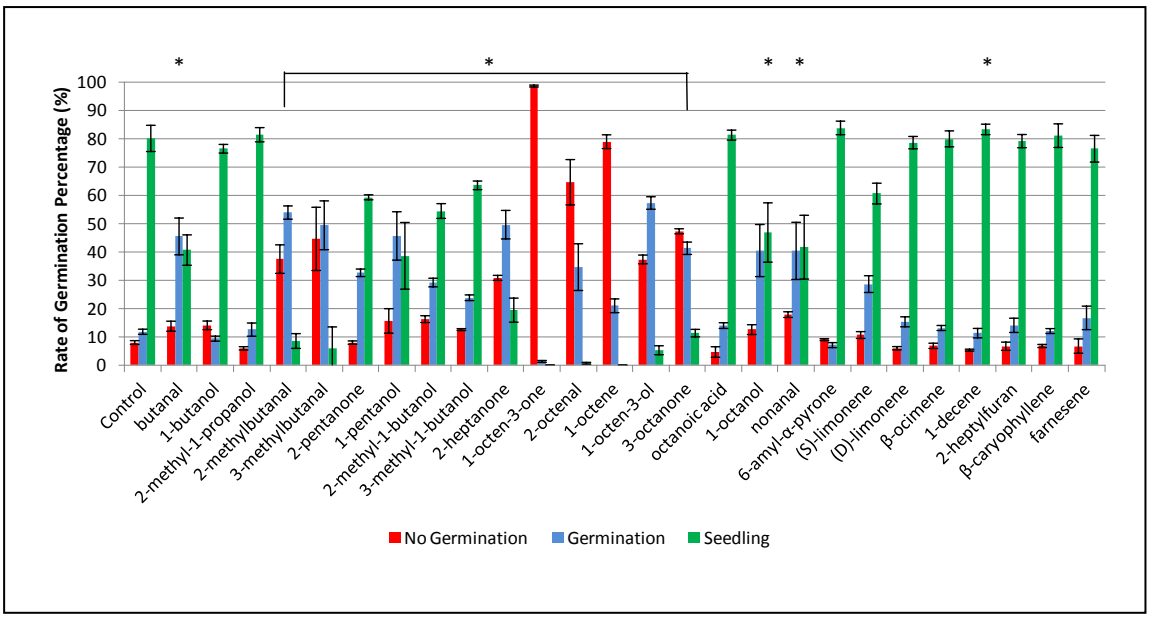


Figure 2. Effects of individual compounds on *Arabidopsis* germination (n = 50, P < 0.001). * denotes significance.

Growth measurements with Arabidopsis plants

Plants treated to individual compounds exhibited various responses, affecting the overall plant size and health of the shoots (Fig. 3). Other visible negative effects include lesions on the leaf or the death of an entire leaf. Plants exposed to 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-heptanone, octanoic acid, limonene, 1-decene, and 2-heptylfuran were larger in size with increased fresh weight (20 - 35% increase) and total chlorophyll content (23 - 50% increase) (Fig. 4). Exposure to 1-pentanol, 1-octen-3-ol, and β-ocimene resulted in a significant reduction in plant fresh weight (13 - 20% decrease) and chlorophyll (20 - 36% decrease). The compounds 1-octen-3-one and nonanal caused complete death in plants in less than 72 hours (P < 0.001).

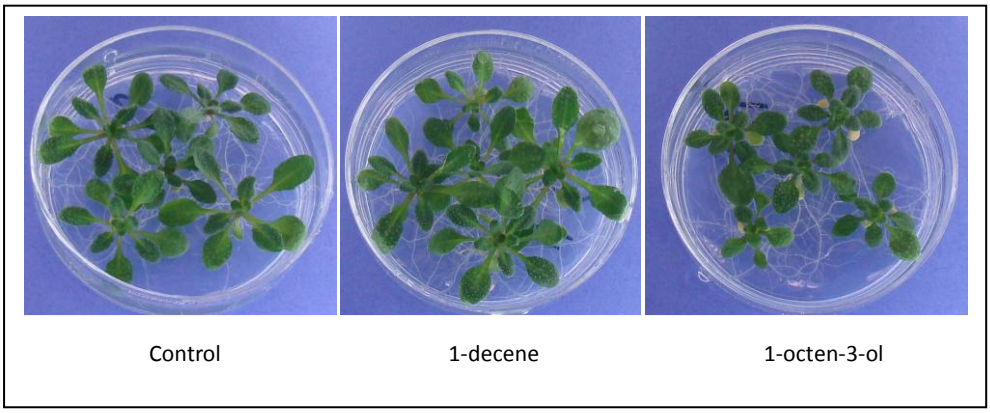


Figure 3. *Arabidopsis* plants exposed to 0.5 µg of individual compound for 72 hours.

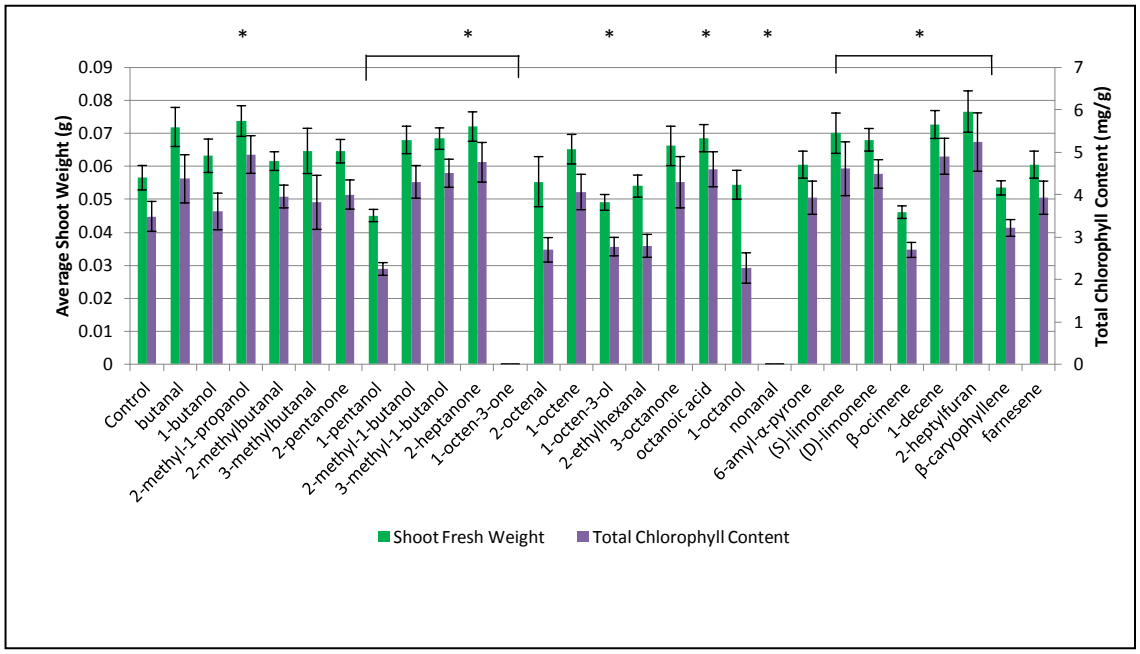


Figure 4. Effects of individual compound on *Arabidopsis* vegetative plant growth. Exposure at 0.5 µg for 72 hours (n = 15, P < 0.001). * denotes significance.

Based on the germination and vegetative assays completed at 0.5 µg concentration (Table 1), we further tested seven compounds at the physiological concentration (10 ng) determined by previous GC-MS analysis of *Trichoderma* isolates

(Fig. 5). We tested commonly detected fungal VOCs: 2-methyl-1-propanol, 2-methyl-1-butanol, 3-methyl-1-butanol, 2-heptanone, and 1-octen-3-ol. We also tested *Trichoderma*-derived compounds, 1-decene and 2-heptylfuran. Three out of seven compounds (3-methyl-1-butanol, 1-decene, and 2-heptylfuran) induced significant increase in fresh weight and total chlorophyll content (ANOVA, $P < 0.01$). Plants exposed to 1-decene had the greatest increase in plant fresh shoot weight (38.9%) and chlorophyll content (67.8%). We also treated plant seeds to 10 ng of compound and grown for 17 days under each condition. Seeds successfully underwent germination, developed into seedlings, and continued to grow. At the end of the 17-day-treatment, plant growth was comparable to the 72 hour exposure. Exposure to 10 ng of 1-decene for 17 days led to the greatest increase in plant biomass (data not shown).

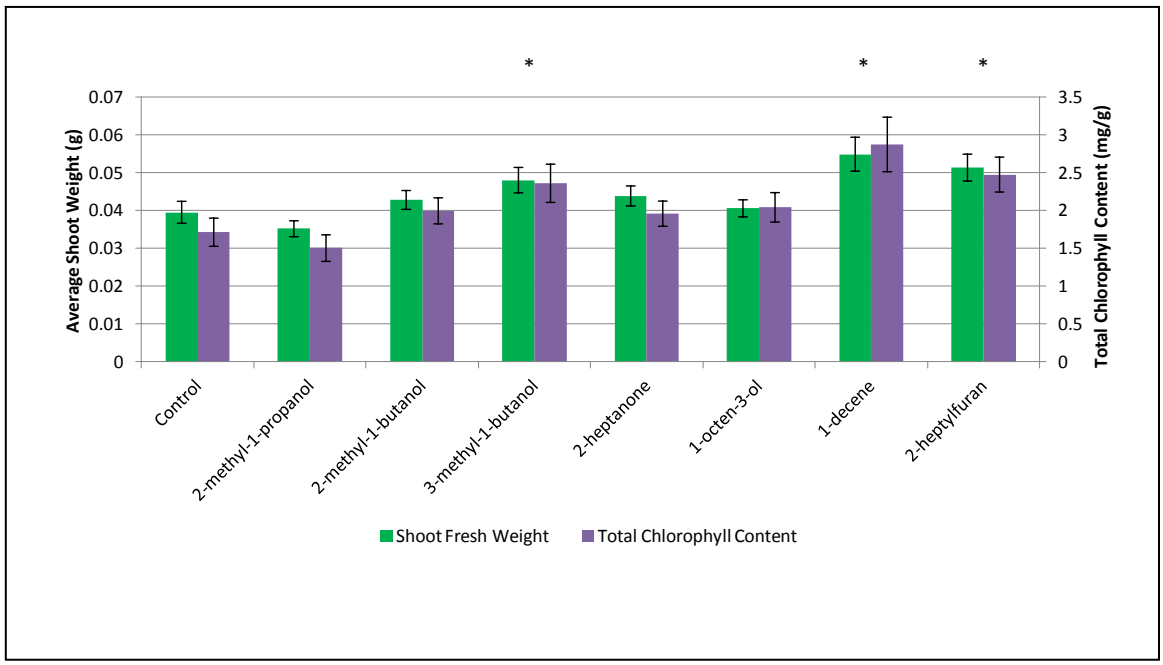


Figure 5. *Arabidopsis* exposed to physiological concentration (10 ng) of individual compounds for 72 hours ($n = 15$, $P < 0.01$).

Profiling differential gene expression of plants exposed to fungal volatile 1-decene using RNA Sequencing

Due to the comprehensive growth promotion observed in plants exposed to 0.5 µg and 10 ng of 1-decene (Fig. 3 and Fig. 5), it was chosen as the compound to use to examine global changes in gene expression following exposure to fungal VOCs. We performed RNA sequencing analysis 72 hours after the application of 1-decene (0.5 µg) to 14-day-old *Arabidopsis* plants. Treatment to 1-decene was compared to control. A >2.0 fold change and p-value of less than 0.05 was regarded as a significant difference. Plants treated with 1-decene differentially changed the transcription of 123 genes. Most genes were down-regulated in plants treated to 1-decene (119 down-regulated and 17 up-regulated). The annotation of the genes that were significantly expressed is listed in Supplemental Table 1. The heatmap generated using the RPKM values of all differentially expressed genes and clustered by biological context is available in Supplemental Figure 1. The differentially expressed gene list included genes that are involved in cellular processes, metabolic processes, and defense responses against pathogens. Some of the most strongly differentially expressed genes include defense and immunity (PP2-A5, WRK18, At5G8120) as well as tissue development (HEC1).

We performed a gene ontology (GO) term enrichment analysis of 117 genes that were differentially expressed. GO analysis was completed for 117 out of 123 genes as six accessions were lost between GO versions. A detailed list of GO analysis is provided in Supplemental Table 2. Using AgriGO software, we identified GO terms enriched for all genes differentially expressed. A total of 110 gene categories were found to be enriched between control and treated plants. The results show that 35% of the total up-

regulated genes are related to cell and cell parts involved in response to hormone and cell wall modification. Among the down-regulated transcripts, there were highly enriched list for relevant terms including 'response to stimulus,' 'response to stress,' and 'defense response' (Table 2). Genes involved in response to stimulus were enriched three folds relative to the background reference. Gene enrichment in response to stimulus included a list of genes involved in response to chemical and organic substances as well as jasmonic acid.

Term	Percentage of Genes	Number of Genes	FDR
response to stimulus	31.62	37	3.10E-24
response to stress	19.66	23	2.50E-19
response to chemical stimulus	19.66	23	1.10E-19
response to organic substance	17.09	20	5.60E-22
defense response	9.40	11	4.70E-14
response to hormone stimulus	8.55	10	5.20E-04
response to jasmonic acid stimulus	5.98	7	2.10E-06

Table 2. Select enriched functional categories of differentially expressed genes ($P < 0.05$). Class identifiers with substantial, low False Discovery Rate ($FDR < 0.01$). Complete list of enriched functional categories is provided in Supplemental Table 2.

5.4 Discussion

We investigated the effects of fungal VOCs that were detected in our previous screen of *Trichoderma* isolates. The bioactivity of these compounds on plant growth is dependent on the developmental stage of the plant, exposure duration, number of compounds present, and the concentration of individual compounds produced by the fungus. We screened 26 standard compounds, C4 to C15 in lengths, including aldehydes, alcohols, ketones, alkenes, esters, and terpenes, using *Arabidopsis thaliana*.

In our study, germination and seedling development was highly sensitive to the presence of C8 hydrocarbons such as 1-octen-3-one, 2-octenal, 1-octene, 1-octen-3-ol, and trans-2-octenal leading to significant inhibition. The production of C8 compounds are characteristic to fungi and alkene volatile, 1-octene is produced by several species of fungi and is a major flavor compound in edible fungi (Sunesson et al., 1995; McAfee and Taylor, 1999; Meruva et al., 2004; Strobel et al., 2008; Ong and Liu, 2011) and *Caragana* shrubs (Zhang et al., 2015). Several compounds derived from 1-octene have been studied extensively for their roles as insect attractants (Wheatley et al., 1997; Davis et al., 2013) and inhibitor or stimulator of microbial growth (Chitarra et al., 2004; 2005; Herrero-Garcia et al., 2011; Berendsen et al., 2013). In plants, much of the work on C8 compounds have been to focused on phytotoxicity and induction of plant defense response (Kishimoto et al., 2007; Splivallo et al., 2007; Lee et al., 2014).

The ketone analog, 1-octen-3-one and secondary alcohol, 1-octen-3-ol are major components of the odor of cultivated mushrooms, mold contamination, and moldy-indoor air. The concentrations of these compounds range from 0.07 µg/L of 1-octen-3-one in grapes and $\geq$ 10 µg/m^3 to 900 µg/m^3 of 1-octen-3-ol from substrates and indoor air (La Guerche et al., 2006; Korpi et al., 2009). Trans-2-octenal is emitted by fungi and plants and has antibacterial activities (Bisignano et al., 2001).

The inhibitory effects of these select C8 compounds on plant growth in our study are consistent with what's been reported in literature (Splivallo et al., 2007; Rezende et al., 2015). In these studies, the exposure to C8 compounds induced significant production of reactive oxygen species (H_2O_2), increasing the activity of antixodiant enzymes in plants. Also, it's been suggested that *Tuber* species suppress plant growth in

truffle growing soils by releasing VOCs. However, the physiological basis for the inhibitory action on germination and seedlings development has not been elucidated.

Several terpenes used in this study are commonly produced by microorganisms and plants. Therefore, it was not surprising to find that the germination rates of seeds treated to terpenes were comparable to controls. The sesquiterpene, β-caryophyllene emitted by plant-growth-promoting fungus, *Talaromyces* sp. is able to enhance the growth of *Brassica* seedlings while increasing resistance to the pathogen, *Colletotrichum* (Yamagiwa et al., 2011**)**. Treating plants with a *Trichoderma*-dervied monoterpene, 6-n-pentyl-2H-pyran-2-one (6-amyl-α-pyrone) at 0.166 - 1 mg/L, induced growth promotion and reduced disease symptoms (Vinale et al., 2008). 'Green leafy' monoterpene volatile such as β-ocimene is found to be emitted by a large number of plants and increases in production during leaf damage (Loughrin et al., 1994; Faldt et al., 2003; Kishimoto et al., 2005). Exposure to this compound leads to an expression of a number of defense-related genes (Arimura et al., 2000). Finally, while C8 alkenes appeared to be inhibitory to seed germination, 1-decene (C10 alkene) induced moderate improvement in seed germination and seedling formation rates.

When 14-day-old *Arabidopsis* plants were treated to individual compounds for 72 hours, the plant response and sensitivity to the compounds were different compared to plant seeds. In general, most short-chain alcohols did not significantly affect plant growth while 9 compounds increased plant biomass. Of these, 1-decene induced the greatest increase in plant biomass and chlorophyll. Relatively high (0.5 µg) and low (10 ng) concentrations of 1-decene led to plant growth promotion in our study. At the higher concentration of 6 mg/l, 1-decene has fungistatic activities (Zuo et al., 2007).

Volatiles, 2-methyl-1-propanol, octanoic acid, limonene, 1-decene, and 2-heptylfuran did not cause adverse effects in seeds and increased growth in older plants. The well known plant monoterpene, limonene, is commonly emitted by microorganisms. The accumulation of this compound in fruits has been shown to be involved in the successful trophic interaction between fruits, insects, and microorganisms (Rodriguez et al., 2011). While limonene appears to stimulate plant growth at 0.5 µg concentration in our study, other studies have reported no effect on seed germination and primary root growth at 10 mM and phytotoxic symptoms at high concentration of 60 ml/l (Abrahim et al., 2000; Ibrahim et al., 2004). *Arabidopsis* seeds treated to octanoic acid alone did not induce significant increase in germination rate; however, vegetative plants grew larger. The short chain saturated fatty acid octanoic acid has been suggested as a sensitivity factor. The compound mimics the effects of pollination and increases ethylene sensitivity in plant tissues (Whitehead and Vaseljevic, 1993; Jager et al., 1996).

The branched alcohol, 2-methyl-1-propanol is one of the dominant microbial VOCs in plant associated microbes. It's been found to be emitted by beneficial microbes including *Bacillus, Pseudomonas, Phoma,* and *Trichoderma* (Farag et al., 2006; Xie et al., 2009; Lee et al., 2015a; Park et al., 2015). Fungi have been shown to emit ≥1.5 $\mu g/m^3$ of 2-methyl-1-proanpol (Korpi et al., 2009). Therefore, it is not surprising this compound can induce plant growth promotion. In our study, 1-octen-3-one was the most inhibitory affecting all aspects of plant growth and development. The majority of the seeds exposed to 1-octen-3-one were unable to germinate and plant exposure resulted in complete the death of the plant in less than 72 hours.

Most studies have focused on understanding volatile-induced systemic resistance in plants. Compounds such as 1-octen-3-ol, 6-amyl-α-pyrone, m-cresol and methyl benzoate induce systemic resistance against pathogens by affecting salicylic acid and jasmonic acid signaling pathways (Kishimoto et al., 2007; Vinale et al., 2008; Naznin et al., 2013). Relatively little is known about the overall gene expression of plants in response to VOCs emitted by microorganisms. One study to date has examined the effects of volatile blends produced by plant growth-promoting rhizobacteria, *Bacillus subtilus* (Zhang et al., 2007). Using a microarray analysis, more than 600 differentially expressed *Arabidopsis* genes were identified and many genes were involved in auxin homeostasis and cell expansion in plants, triggering growth promotion.

We completed a RNA sequencing analysis of *Arabidopsis* shoots treated by fungal VOC, 1-decene, which induced greatest effects in plant growth and development. A total of 123 differentially expressed *Arabidopsis* genes in response to 1-decene exposure were identified. Six genes associated with growth, involved in response to hormone and cell wall modification, were up-regulated. They are: cell wall modification gene involved in rigidity (AT2G43870), transcription factor (AT5G67060), SAUR25 auxin-responsive (AT4G13790), ARGOS-like (AT2G44080), and unknown proteins (AT5G57760) and all are inducible by auxin (Goda et al., 2004; Zhang et al., 2007; Lee et al., 2009; Chapman et al., 2012). MYB122 (AT1G74080) which is involved in the biosynthesis of indolic glucosinolates following environmental challenges and functions as stimulators of auxin biosynthesis (Frerigmann and Gigolashvili, 2014) was also up-regulated. The phytohormone auxin is known to be important in plant growth including leaf growth, shoot elongation, branching of roots, and meristem activity (Zhao, 2011).

We found down-regulation of genes involved in stress (23 genes) and defense responses (11 genes). WRKY transcription factors are important in the regulation of genes associated with plant defense responses. WRKY18, WRKY40, and WRKY58 and several disease resistance proteins were all down-regulated in plants exposed to 1-decene. The gene related to jasmonic acid (JA) biosynthesis LOX4 (AT1G72520) and a repressor of JA, JAZ7 (AT2G34600), were decreased. JA is involved in plant immunity and resistance to abiotic stresses. It also influences the expression of defense genes (Farmer et al., 2003).

A study examining the direct interactions between *Trichoderma* and plants showed that *Arabidopsis* defense-related genes that are mediated by salicylic acid (SA) and jasmonic acid (JA) were down-regulated allowing the fungi to colonize plant roots (Moran-Diez et al., 2012). Interaction with *Trichoderma* has been shown to modify WRKY transcription factors to modulate the expression of the JAZ repressor genes and defense response genes (Brotman et al., 2013). *Arabidopsis* exposed to growth promoting bacterium caused a significant number of genes to be down-regulated including genes associated with stress response, and response to biotic and abiotic stimulus (Poupin et al., 2013). It appears that beneficial microorganisms, when in direct contact with plants, will reduce expression of defense and stress response genes during the initial establishment of host plants. It is possible that prior to direct interaction between fungus and plant, *Trichoderma* spp. may release VOCs as early signals to reduce plant immunity to allow successful colonization of the plant. Further study is needed to determine if exposure to 1-decene will adversely affect plant immunity and defense against pathogens.

In summary, this study showed that an individual fugal VOC is able to stimulate *Arabidopsis* growth. Nine compounds are able to positively affect seed germination and plant development. To our knowledge, this is the first study quantifying global gene expression of plants treated to an individual fungal volatile organic compound using RNA sequencing. Of the 123 genes, 17 genes were up-regulated and many have roles in growth and biological processes. From the gene expression data, we identified several auxin-induced genes leading to larger plant biomass. We also unexpectedly found that a large number of genes in defense response as well as response to chemical and organic stimuli were down-regulated. Studies have reported several microbial VOCs that induce activation of defense related genes in plants; however, they differ in the structure of the compound, concentrations, and exposure conditions. Future studies should consider examining genes targeted specifically to hormone related growth responses, in auxin and JA actions, to screen and assess different fungal VOCs. The candidate 1-decene-responsive genes identified in this study can be used to devise future experiments with the intent of shedding light into specific pathways that may be affected by VOC exposure.

REFERENCES

Abrahim, D., Braguini, W.L., Kelmer-Brancht, K., Ishii-Iwamoto, E.L. (2000). Effects of four monoterpenes on germination, primary root growth, and mitochondrial respiration of maize. Journal of Chemical Ecology. 26: 611-624.

Arimura, G., Ozawa, R., Shimoda, T., Nishioka, T., Boland, W. and Takabayashi, J. (2000). Herbivory-induced volatiles elicit defense gene in lima bean leaves. Nature. 406: 512-515.

Berendsen, R.L., Kalkhove, S.I., Lugones, L.G., Baars, J.J., Wösten, H.A., Bakker, P.A. (2013). Effects of the mushroom-volatile 1-octen-3-ol on dry bubble disease. Applied Microbiology and Biotechnology. 97: 5535-5543.

Bisignano, G., Lagana, M.G., Trombetta, D., Arena, S., Nostro, A., Uccella, N., Mazzanti, G., Saija, A. (2001). In vitro antibacterial activity of some aliphatic aldehydes from *Olea europaea* L. FEMS Microbiology Letters. 198: 9-13.

Brotman, Y., Landau, U., Cuadros-Inostroza, A., Takayuki, T., Fernie, A.R., Chet, I., Viterbo, A., Willmitzer, L. (2013). *Trichoderma*-plant root colonization: escaping early palnt defense responses and activation of the antioxidant machinery for saline stress tolerance. PLoS Pathogens. 9: e1003221.

Chang, C.-W., Chang, H.-S., Cheng, M.-J., Liu, T.-W., Hsieh, Y., Yuan, G.-F., Chen, I.-S. (2014). Inhibitory effects of constituents of an endophytic fungus *Hypoxylon investiens* on nitric oxide and interleukin-6 production in RAW264.7 macrophages. Chemistry & Biodiversity. 11: 949-961.

Chapman, E.J., Greenham, K., Castillejo, C., Sartor, R., Bialy, A., Sun, T.-P., Estelle, M. (2012). Hypocotyl transcriptome reveals auxin regulation of grow-promoting genes through GA-dependent and independent pathways. PLoS ONE. 7(5): e36210. doi:10. 1371/journal.pone.0036210.

Chitarra, G.S., Abee, T., Rombouts, F.M., Posthumus, M.A., Dijksterhuis, J. (2004). Germination of *Penicillium paneum* conidia is regulated by 1-octen-3-ol, a volatile self-inhibitor. Applied and Environmental Microbiology. 70: 2823-2829.

Chuankun, X., Minghe, M., Leming, Z., Keqin, Z. (2004). Soil volatile fungistasis and volatile fungistatic compounds. Soil Biology and Biochemistry. 36: 1997-2004.

Claeson, A.S., Levin, J.O., Blomquist, G., Sunesson, A.L. (2002). Volatile metabolites from microorganisms grown on humid building materials and synthetic media. Journal of Environmental Monitoring. 4: 667-672.

Davis, T.S., Crippen, T.L., Hofstetter, R.W., Tomberlin, J.K. (2013). Microbial volatile emissions as insect semiochemicals. Journal of Chemical Ecology. 39: 840-859.

Faldt, J., Arimura, G., Gershenzon, J., Takabayashi, J., Bohlmann, J. (2003). Functional identification of AtTPS03 as (*E*)-β-ocimene synthase: a monoterpene synthase catalyzing jasmonate- and wound- induced volatile formation in *A. thaliana*. Planta. 216: 745-751.

Farag, M.A., Ryu, C.M., Sumner, L.W., Pare, P.W. (2006). GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry. 67: 2262-2268.

Farmer, E.E., Alméras, E., Krishnamurthy, V. (2003). Jasmonates and related oxylipins in plant responses to pathogenesis and herbivory. Current Opinion in Plant Biology. 6: 372-378.

Frerigmann, H., Gigolashvili, T. (2014). MYB34, MYB51, and MYB122 distinctly regulate indolic glucosinolate biosynthesis in *Arabidopsis thaliana*. Molecular Plant. 7: 814-828.

Goda, H., Sawa, S., Asami, T., Fujioka, S., Shimada, Y., Yoshida, S. (2004). Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in *Arabidopsis*. Plant Physiology. 134: 1555-1573.

Herrero-Garcia, E., Garzia, A., Cordobés, S., Espeso, E.A., Ugalde, U. (2011). 8-Carbon oxylipins inhibit germination and growth, and stimulate aerial conidiation in *Aspergillus nidulans*. Fungal Biology. 115: 393-400.

Hung, R., Lee, S., Bennett, J.W. (2013). *Arabidopsis thaliana* as a model system for testing the effect of *Trichoderma* volatile organic compounds. Fungal Ecology. 6: 19-26.

Ibrahim, M.A., Oksanen, E.J., Holopainen, J.K. (2004). Effects of limonene on the growth and physiology of cabbage (*Brassica oleracea* L) and carrot (*Daucus carota* L) plants. Journal of the Science of Food and Agriculture. 84: 1319-1326.

Insam, H., Seewald, S.A. (2010). Volatile organic compounds (VOCs) in soils. Biology and Fertility of Soils. 46: 199-213.

Jager, A.K., Strydom, A., van Staden, J. (1996). The effect of ethylene, octanoic acid and a plant-derived smoke extract on the germination of light-sensitive lettuce seeds. Plant Growth Regulation. 19: 197-201.

Kishimoto, K., Matsui, K., Ozawa, R. (2007). Volatile 1-octen-3-ol induces a defense response in *Arabidopsis thaliana*. Journal of General Plant Pathology. 73: 35-37.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J. (2005). Volatile C6-aldehydes and Allo-ocimene activate defense genes and induce resitance against *Botrytis cinerea* in *Arabidopsis thaliana*. Plant and Cell Physiology. 46: 1093-1102.

Korpi, A., Jarnberg, J., Pasanen, A.L. (2009). Microbial volatile organic compounds. Critical Reviews in Toxicology. 39: 139-193.

Kudalkar, P., Strobel, G., Hasan, S.R.U., Geary, G., Sears, J. (2012). *Muscodor sutura* a novel endophytic fungus with volatile antibiotic activities. Mycoscience. 53: 319-325.

La Guerche, S., Dauphin, B., Pons, M., Blancard, D., Darriet, P. (2006). Characterization of some mushroom and earth off-odors microbially induced by the development of rot on grapes. Journal of Agricultural and Food Chemistry. 29: 9193-9200.

Lee, D.J., Park, J.W., Lee, H.W., Kim, J. (2009). Genome-wide analysis of auxin-response transcriptome downstream of iaa1 and its expression analysis reveal the diversity and complexity of auxin-regulated gene expression. Journal of Experimental Botany. 60: 3935-3957.

Lee, S., Hung, R., Schink, A., Mauro, J., Bennett, J.W. (2014). *Arabidopsis thaliana* for testing the phytotoxicity of volatile organic compounds. Plant Growth Regulation. 74: 177-186.

Lee, S., Hung, R., Yap, M., Bennett, J.W. (2015a). Age matters: the effects of volatile organic compounds emitted by *Trichoderma atroviride* on plant growth. Archives of Microbiology. 197: 723-727.

Lee, S., Hung, R., Yin, G., Klick, M.A., Grimm, C., Bennett, J.W. (2015b). Strain specific interaction between *Aspergillus versicolor* and plants through volatile organic compounds. *Under Review.*

Lee, S., Hung, R., Behringer, G., Bennett, J.W. Analysis of volatile organic compounds emitted by *Trichoderma* species and volatile-mediated plant growth. *In Preparation*.

Loughrin, J.H., Manukian, A., Heath, R.R., Turlings, T.C., Tumlinson, J.H. (1994). Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plant. Proceedings of the National Academy of Sciences. 91: 11836-11840.

Matysik, S., Herbarth, O., Mueller, A. (2009). Determination of microbial volatile organic compounds (MVOCs) by passive sampling onto charcoal sorbents. Chemosphere. 76: 114-119.

McAfee, B.J., Taylor, A. (1999). A review of the volatile metabolites of fungi found on wood substrates. Natural Toxins. 7: 283-303.

Meruva, N.K., Penn, J.M., Farthing, D.E. (2004). Rapid identification of microbial VOCs from toabcco molds using closed-loop stripping and gas chromatography/time-of-flight mass spectrometry. Journal of Industrial Microbiology & Biotechnology. 31: 482-488.

Mitchell, A.M., Strobel, G.A., Moore, E., Robison, R., Sears, J. (2010). Volatile antimicrobials from *Muscodor crispans*, a novel endophytic fungus. Microbiology. 156: 270-277.

Moran-Diez, M., Trushina, N., Lamdan, N.L., Rosenfelder, L., Mukherjee, P.K., Kenerley, C.M., Horwitz, B.A. (2015). Host-specific transcriptomic pattern of *Trichoderma virens* during interaction with maize or tomato roots. BMC Genomics. 16: 8. doi.10.1186/s12864-014-12083.

Naznin, H.A., Kimura, M., Miyazawa, M., Hyakumachi, M. (2013). Analysis of volatile organic compounds emitted by plant growth-promoting fungus *Phoma* sp. GS8-3 for growth promotion effects on tobacco. Microbes and Environments. 28: 42-49.

Ong, P.K.C., Liu, S.Q. (2011). Flavor and sensory characteristics of vegetables. In: Sinha, N., Kui, Y.H., Evranuz, O. (Ed.) Handbook of vegetables and vegetable processing. Wiley-Blackwell Publishing. Pgs. 73-74.

Park, Y.-S., Dutta, S., Ann, M., Raaijmakers, J.M., Kyungseok, P. (2015). Promotion of plant growth by *Pseudomonas fluorescens* strain SS101 via novel volatile organic compounds. Biochemical and Biophysical Research Communications. 2: 361-365.

Penuelas, J., Asensio, D., Thol, D., Wenke, K., Rosenkranz, M., Piechulla, B., Schnitzler, J.P. (2014). Biogenic volatile emissions from the soil. Plant, Cell and Environment. 37: 1866-1891.

Poupin, M.J., Timmermann, T., Vega, A., Zuniga, A., Gonzalez, B. (2013). Effects of the plant growth-promoting bacterium *Burkholderia phytofirmans* PsJN throughout the life cycle of *Arabidopsis thaliana*. PLoS ONE. 8: e69435.

Rezende, D.C., Fialho, M.B., Brand, S.C., Blumer, S., Pascholati, S.F. (2015). Antimicrobial activity of voaltile organic compounds and their effect on lipid peroxidation and electrolyte loss in *Colletotrichum gloeosporioides* and *Colletotrichum acutatum* mycelia. African Journal of Microbiology Research. 9: 1527-1535.

Rodriguez, A., Andres, V.S., Cervera, M., Redondo, A., Alquezar, B., Shimada, T., Gadea, J., Rodrigo, M.J., Zacarias, L., Palou, L., Lopez, M.M., Castanera, P., Pena, L. (2011). Terpene down-regulation in orange reveals the role of fruit aromas in mediating interactions with insect herbivores and pathogens. Plant Physiology. 156: 793-802.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wie, H.-X., Pare, P.W., Kloepper, J.W. (2003). Bacterial volatiles promote growth of *Arabidopsis*. Proceedings of the National Academy of Sciences. 100: 4927-4932.

Splivallo, R., Novero, M., Bertea, C.M., Bossi, S., Bonfante, P. (2007). Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist. 175: 417-424.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C. (2001). Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Microbiology. 147: 2943-2950.

Strobel, G.A., Knighton, B., Kluck, K., Ren, Y., Livinghouse, T., Griffin, M., Spakowicz, D., Sears, J. (2008). The production of myco-diesel hydrocarbons and their derivatives by the endophytic fungus *Gliocladium roseum* (NRRL 50072). Microbiology. 154: 3319-3328.

Sunesson, A.-L., Vaes, W.H.J., Nilsson, C.A., Blomquist, G., Andersson, B., Carlson, R. (1995). Identification of volatile metabolites from five fungal species cultivated on two media. Applied and Environmental Microbiology. 61: 2911-2918.

Vespermann, A., Kai, M., Piechulla, B. (2007). Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Applied Environmental Microbiology. 73: 5639-5641.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Woo, S.L., Lorito, M. (2008). *Trichoderma*-plant pathogens interactions. Soil Biology and Biochemistry. 40: 1-10.

Wheatley, R., Hackett, C., Bruce, A., Kundzewicz, A. (1997). Effect of substrate composition on production of volatile organic compounds from *Trichoderma* spp. inhibitory to wood decay fungi. International Biodeterioration & Biodegradation. 39: 199-205.

Whitehead, C.S., Bosse, C.A. (1991). The effect of ethylene and short-chain saturated fatty acids on ethylene sensitivity and binding in ripening bananas. Journal of Plant Physiology. 137: 358-362.

Whitehead, S.C., Vaseljevic, D. (1993). Role of short-chain saturated fatty acids in control of ethylene sensitivity in senescing carnation flowers. Physiologia Plantarum. 88: 250-342.

Xie, X., Zhang, H., Pare, P.W. (2009). Sustained growth promotion in *Arabidopsis* with long-term exposure to the beneficial soil bacterium *Bacillus subtilis* (GB03). Plant Signaling & Behavior. 4: 948-953.

Yamagiwa, Y., Inagaki, Y., Ichinose, Y., Toyoda, K., Hyakumachi, M., Shiraishi, T. (2011). *Talaromyces wortmannii* FS2 emits β-caryophyllene, which promotes plant growth and induces resistnace. Journal of General Plant Pathology. 77: 336-341.

Zhang, Y.-R., Wang, R., Yu, L.-F., Lu, P.-F., Luo, Y.-Q. (2015). Identification of *Caragana* plant volatiles, overlapping profiles, and olfactory attraction to *Chlorophorus caragana* in the laboratory. Journal of Plant Interactions. 10: 41-50.

Zhang, H., Kim, M.-S., Krishnamachari, V., Payton, P., Sun, Y., Grimson, M., Farag, M. A., Ryu, C.-M., Allen, R., Melo, I.S., Pare, P.W. (2007). Rhizobacterial volatile emissions regulate auxin homeostasis and cell expansion in *Arabidopsis*. Planta. 226: 839-851.

Zhao, Y. (2011). Auxin biosynthesis and its role in plant development. Annual Review of Plant Biology. 61: 49-64.

Zou, C.-S., Mo, M.-H., Gu, Y.-Q., Zhou, J.-P., Zhang, K.-Q. (2007). Possible contributions of volatile-producing bacteria to soil fungistasis. Soil Biology & Biochemistry. 39: 2371-2379.

Supplemental Table 1. List of differentially expressed genes and fold changes in *Arabidopsis* shoots 72 hours after treatment to 1-decene.

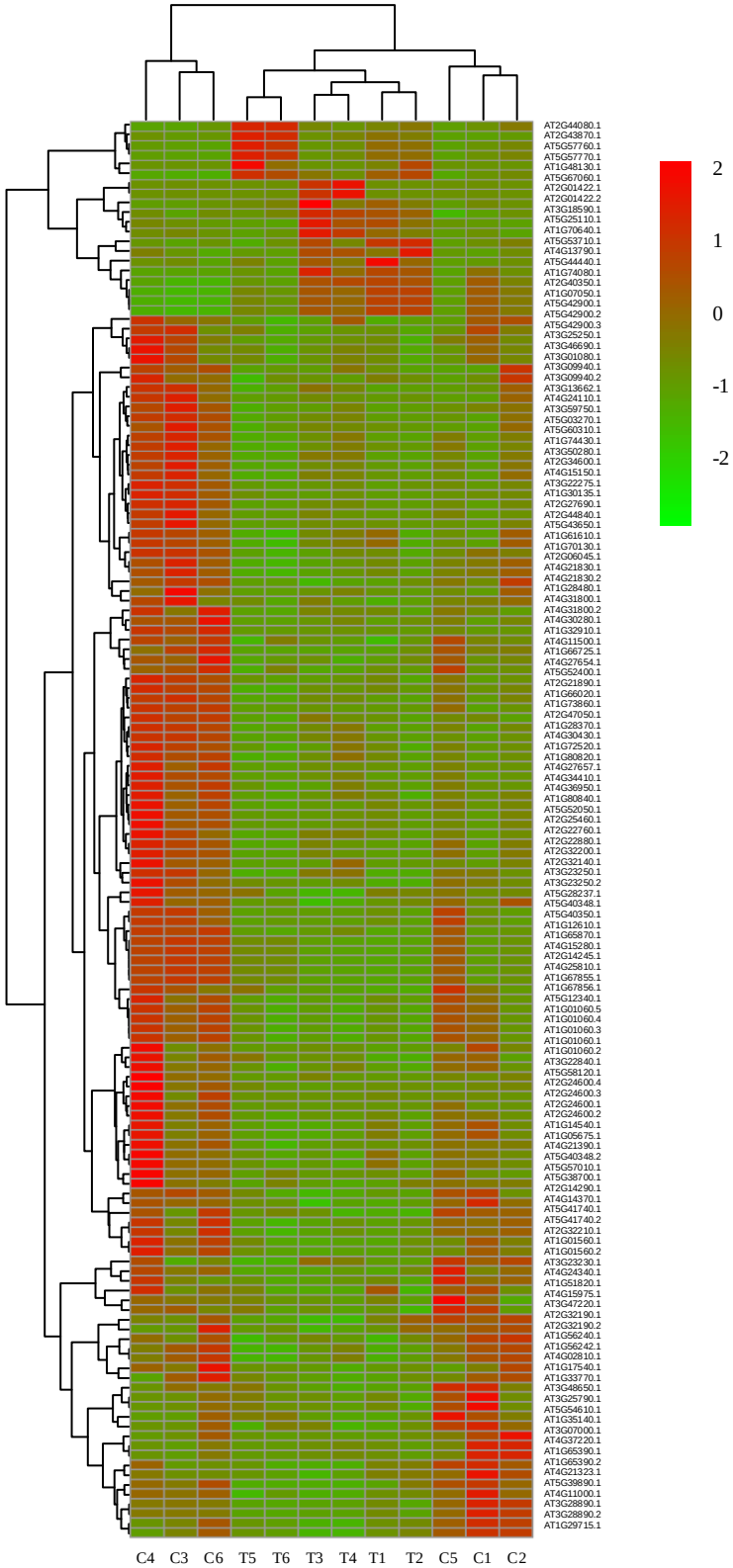
Accession ID	Name	Fold Change (log2)	P-value	Annotation or Description
AT2G14290		-5.15220886	0.000590898	F-box family protein with a domain of unknown function (DUF295)
AT5G52400	CYP715A1	-4.587047965	0.001521551	cytochrome P450, family 715, subfamily A, polypeptide 1
AT5G38700		-4.566863375	0.041204851	uncharacterized protein
AT1G66020		-4.44131905	0.038586048	terpene cyclase, C1 domain-containing protein
AT3G29633		-3.884314653	0.003419721	uncharacterized protein
AT1G17540		-3.49704474	0.012575135	Protein kinase protein with adenine nucleotide alpha hydrolases-like domain
AT5G40350	MYB24	-3.030984562	0.000389847	myb domain protein 24
AT2G22760		-2.925434482	0.011443957	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT5G12340		-2.903749005	0.000300462	uncharacterized protein
AT1G70130		-2.869972708	0.019551025	Concanavalin A-like lectin protein kinase family protein
AT1G14540	PER4	-2.860989151	0.005012021	Peroxidase superfamily protein
AT5G43650	BHLH92	-2.814100249	0.004643382	superfamily protein; transcription factor bHLH92
AT1G32910		-2.725025504	0.022580467	HXXXD-type acyl-transferase family protein
AT1G80820	ATCCR2	-2.66882551	0.001883679	cinnamoyl coa reductase
AT3G23250	MYB15	-2.665455268	0.007500745	myb domain protein 15
AT5G40348		-2.572076311	0.000878556	NA
AT2G06045		-2.479157866	0.033099843	copia-like retrotransposon family, has a 2.2e-12 P-value blast match to GB:AAC02672 polyprotein
AT4G27654		-2.444489967	0.009891875	uncharacterized protein
AT3G23230	TDR1	-2.442130851	0.024282039	Integrase-type DNA-binding superfamily protein
AT2G32140		-2.336772779	0.002918284	transmembrane receptors
AT4G30430	TET9	-2.139916562	0.032860309	tetraspanin9
AT1G61610		-2.137230981	0.018384683	S-locus lectin protein kinase family protein
AT3G13662		-2.11495429	0.034428398	Disease resistance-responsive (dirigent-like protein) family protein
AT4G36950	MAPKKK 21	-2.059871179	0.040970045	mitogen-activated protein kinase kinase kinase 21
AT4G14370		-2.052720435	0.00206718	Disease resistance protein (TIR-NBS-LRR class) family
AT1G72520	LOX4	-2.050857193	0.000992484	lipoxygenase 4; PLAT/LH2 domain-containing lipoxygenase family protein
AT1G12610	DDF1	-1.948782993	0.049245937	DWARF AND DELAYED FLOWERING 1; Integrase-type DNA-binding superfamily protein
AT1G30135	JAZ8	-1.928144393	0.007832582	jasmonate-zim-domain protein 8
AT2G44840	ERF13	-1.912261505	0.008841844	ethylene-responsive element binding factor 13
AT1G56242		-1.896094965	9.97E-05	NA
AT2G25460		-1.886620487	0.009768357	uncharacterized protein
AT5G28237		-1.867641936	0.0440885	Pyridoxal-5'-phosphate-dependent enzyme family protein
AT1G28480	GRX480	-1.862816408	0.000142569	glutaredoxin-C9; Thioredoxin superfamily protein

AT4G15150		-1.836929029	0.026861412	glycine-rich protein
AT1G56240	PP2-B13	-1.82245289	9.27E-05	phloem protein 2-B13
AT3G22275		-1.813673658	0.03654673	uncharacterized protein
AT2G34600	JAZ7	-1.793942202	0.006876353	jasmonate-zim-domain protein 7
AT2G32200		-1.787394398	0.008034543	uncharacterized protein
AT4G34410	RRTF1	-1.721735289	0.020956894	redox responsive transcription factor 1
AT1G66725		-1.70998212	0.022314635	NA
AT5G60310		-1.696741199	0.001597332	Concanavalin A-like lectin protein kinase family protein
AT5G57010		-1.642208193	0.033126635	calmodulin-binding family protein
AT2G22880		-1.637010027	0.008051581	VQ motif-containing protein
AT4G11500		-1.586606224	0.016386039	NA
AT1G28370	ERF11	-1.583216842	0.006116536	ERF domain protein 11
AT1G01060	LHY	-1.567891616	0.001276294	LATE ELONGATED HYPOCOTYL; Homeodomain-like superfamily protein
AT4G24340		-1.555612035	0.011836967	Phosphorylase family protein
AT3G01080	WRKY58	-1.544349398	0.000643425	WRKY DNA-binding protein 58
AT3G09940	MDHAR	-1.535205686	0.02149532	monodehydroascorbate reductase
AT4G21390	B120	-1.529799714	0.002621057	S-locus lectin protein kinase family protein
AT4G30280	XTH18	-1.520967054	5.98E-07	endotransglucosylase/hydrolase 18, xyloglucan
AT2G27690	CYP94C1	-1.497439449	0.02495703	cytochrome P450, family 94, subfamily C, polypeptide 1
AT1G67855		-1.48182461	0.001729528	uncharacterized protein
AT5G03270	LOG6	-1.472186061	0.037837156	lysine decarboxylase family protein
AT1G67856		-1.439354317	0.002047791	RING/U-box superfamily protein
AT5G14110		-1.435591597	0.006814705	uncharacterized protein
AT4G27657		-1.42602718	0.027940953	uncharacterized protein
AT3G46690		-1.405581096	0.00465508	UDP-Glycosyltransferase superfamily protein
AT1G65390	PP2-A5	-1.375564527	3.70E-07	phloem protein 2 A5
AT1G29715		-1.37238623	0.047115031	NA
AT2G32210		-1.346883599	0.000970339	uncharacterized protein
AT2G14245		-1.346451823	0.032654007	NA
AT1G01560	MPK11	-1.343710054	0.011418592	MAP kinase 11
AT4G15280	UGT71B5	-1.320769595	0.001497335	UDP-glucosyl transferase 71B5
AT3G22840	ELIP1	-1.31999901	0.001310262	Chlorophyll A-B binding family protein
AT1G65870		-1.319690449	0.010670344	Disease resistance-responsive (dirigent-like protein) family protein
AT3G50280		-1.28656297	0.048760334	uncharacterized acetyltransferase
AT1G51820		-1.278631286	2.33E-06	putative Leucine-rich repeat protein kinase family protein
AT3G25250	AGC2-1	-1.272272757	0.008995494	AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase family protein
AT3G43850		-1.254759204	0.006994614	uncharacterized protein
AT5G41080	GDPD2	-1.25076739	0.045000385	PLC-like phosphodiesterases superfamily protein
AT2G35710	PGSIP7	-1.240295112	0.007405339	Nucleotide-diphospho-sugar transferases superfamily protein
AT1G35140	PHI-1	-1.239032606	0.00306719	Phosphate-responsive 1 family protein
AT2G32190		-1.232347489	0.006551713	uncharacterized protein
AT5G03360		-1.214272318	0.000170585	DC1 domain-containing protein
AT4G25810	XTR6	-1.210863287	3.94E-05	xyloglucan endotransglycosylase 6
AT1G05675		-1.208432683	0.010539931	UDP-Glycosyltransferase superfamily protein

AT3G59750		-1.192824422	0.027569522	Concanavalin A-like lectin protein kinase family protein
AT1G33770		-1.182137684	0.006826779	Protein kinase superfamily protein
AT3G25790		-1.182052547	0.048060433	myb-like transcription factor family protein
AT1G73860		-1.166177049	0.042771247	P-loop containing nucleoside triphosphate hydrolases superfamily protein
AT2G21890	CAD3	-1.165151897	0.029694877	cinnamyl alcohol dehydrogenase homolog 3
AT4G21830	MSRB7	-1.16401084	0.040739336	methionine sulfoxide reductase B7
AT4G31800	WRKY18	-1.159230872	2.40E-05	WRKY DNA-binding protein 18
AT4G15975		-1.112522251	0.000792599	RING/U-box superfamily protein
AT1G02700		-1.108162721	0.004954772	uncharacterized protein
AT5G01040	LAC8	-1.103176359	0.014159519	laccase 8
AT1G80840	WRKY40	-1.101536002	0.018045687	WRKY DNA-binding protein 40
AT5G41740		-1.098166567	0.000464291	Disease resistance protein (TIR-NBS-LRR class) family
AT5G52050		-1.09164301	0.040534364	MATE efflux family protein
AT5G58120		-1.081653582	9.16E-05	Disease resistance protein (TIR-NBS-LRR class) family
AT3G48650		-1.076435869	0.015086575	NA
AT1G74430	MYB95	-1.04955225	0.024228678	myb domain protein 95
AT2G24600		-1.048712671	1.63E-06	Ankyrin repeat family protein
AT4G21323		-1.045498417	0.016790639	Subtilase family protein
AT2G47050		-1.043221779	0.008277664	Plant invertase/pectin methylesterase inhibitor superfamily protein
AT3G28890	RLP43	-1.041373493	0.045889306	receptor like protein 43
AT3G07000		-1.039730379	0.025646255	Cysteine/Histidine-rich C1 domain family protein
AT3G47220	PLC9	-1.031209812	0.012137032	phosphatidylinositol-speciwc phospholipase C9
AT4G11000		-1.028593351	0.004905758	Ankyrin repeat family protein
AT1G25400		-1.018281727	1.33E-05	uncharacterized protein
AT5G54610	ANK	-1.013932944	0.002937604	ankyrin
AT4G02810	FAF1	-1.006892378	0.045282447	Protein of unknown function (DUF3049)
AT5G39890		-1.002997903	0.041718834	Protein of unknown function (DUF1637)
AT4G37220		-1.002774007	0.001034721	Cold acclimation protein WCOR413 family
AT4G24110		-1.001377708	0.038765459	uncharacterized protein
AT5G42900	COR27	1.034601468	0.01067489	cold regulated gene 27 basic helix-loop-helix (bHLH) DNA-binding
AT5G25110	CIPK25	1.03864417	0.000342649	CBL-interacting protein kinase 25
AT5G44440		1.108657771	0.011871498	FAD-binding Berberine family protein
AT1G07050		1.175385084	0.020384168	CCT motif family protein
AT2G44080	ARL	1.275199387	0.039958288	ARGOS-like
AT1G48130	PER1	1.49704474	0.023507449	1-cysteine peroxiredoxin 1
AT4G13790	SAUR25	1.561789141	0.033055835	SAUR-like auxin-responsive protein family
AT5G67060	HEC1	1.687949909	5.52E-06	basic helix-loop-helix (bHLH) DNA-binding superfamily protein
AT5G53710		1.741074361	0.028032423	uncharacterized protein
AT1G70640		1.789344567	0.017043458	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein
AT5G57760		1.917795355	0.00915705	uncharacterized protein
AT5G57770		1.956990666	0.008685932	uncharacterized protein
AT1G74080	MYB122	2.018523579	0.012105299	myb domain protein 122
AT2G43870		2.020402243	0.005687605	Pectin lyase-like superfamily protein

AT2G40350		2.420911309	0.024918174	Integrase-type DNA-binding superfamily protein
AT3G18590	ENODL5	3.154570318	0.016186346	early nodulin-like protein 5
AT2G01422		4.887147659	0.026425207	NA

Supplemental Figure 1. Heatmap showing differential expression of 123 genes between control (C) and treated (T) to 1-decene.



Supplemental Table 2. Complete list of enriched functional categories of *Arabidopsis*. (bgitem: query item number in *Arabidopsis* genome; bgtotal = total annotated item number in *Arabidopsis* genome)

GO_acc	Term type	Term	-queryitem -querytotal -bgitem -bgtotal -p-value -FDR	Entries
GO:0050896	P	response to stimulus	37 117 985 31819 9.10E-27 3.10E-24	AT4G31800.1 ,AT1G28370.1 ,AT3G23230.1 ,AT2G34600.1 ,AT1G01060.1 ,AT5G58120.1 ,AT2G44840.1 ,AT1G12610.1 ,AT1G80840.1 ,AT4G37220.1 ,AT1G28480.1 ,AT1G14540.1 ,AT4G13790.1 ,AT1G80820.1 ,AT3G09940.1 ,AT3G22840.1 ,AT3G23250.1 ,AT2G32140.1 ,AT1G48130.1 ,AT1G65870.1 ,AT2G44080.1 ,AT1G01560.1 ,AT5G52050.1 ,AT4G34410.1 ,AT2G40350.1 ,AT1G17540.1 ,AT4G14370.1 ,AT5G41740.1 ,AT1G72520.1 ,AT1G74430.1 ,AT1G65390.1 ,AT5G54610.1 ,AT4G15975.1 ,AT5G40350.1 ,AT4G21830.1 ,AT3G01080.1 ,AT3G13662.1
GO:0050789	P	regulation of biological process	30 117 767 31819 4.40E-22 6.30E-20	AT1G66725.1 ,AT3G28890.1 ,AT4G31800.1 ,AT3G07000.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT1G01060.1 ,AT5G58120.1 ,AT2G44840.1 ,AT1G80840.1 ,AT5G67060.1 ,AT3G09940.1 ,AT3G23250.1 ,AT2G32140.1 ,AT1G28370.1 ,AT4G14370.1 ,AT2G44080.1 ,AT1G01560.1 ,AT4G34410.1 ,AT2G40350.1 ,AT3G25790.1 ,AT5G41740.1 ,AT1G28480.1 ,AT1G74430.1 ,AT3G47220.1 ,AT1G12610.1 ,AT3G01080.1 ,AT5G40350.1 ,AT2G22760.1
GO:0010033	P	response to organic substance	20 117 210 31819 5.60E-22 6.30E-20	AT1G80840.1 ,AT5G40350.1 ,AT4G31800.1 ,AT2G44080.1 ,AT4G15975.1 ,AT4G13790.1 ,AT1G01560.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G09940.1 ,AT5G54610.1 ,AT3G23250.1 ,AT2G34600.1 ,AT1G01060.1 ,AT1G28370.1 ,AT2G44840.1 ,AT1G28480.1 ,AT1G48130.1 ,AT4G37220.1 ,AT1G74430.1
GO:0065007	P	biological regulation	31 117 882 31819 1.60E-21 1.40E-19	AT1G66725.1 ,AT3G28890.1 ,AT4G31800.1 ,AT3G07000.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT1G01060.1 ,AT5G58120.1 ,AT2G44840.1 ,AT1G80840.1 ,AT5G67060.1 ,AT3G09940.1 ,AT3G23250.1 ,AT2G32140.1 ,AT4G21323.1 ,AT1G28370.1 ,AT4G14370.1 ,AT2G44080.1 ,AT1G01560.1 ,AT4G34410.1 ,AT2G40350.1 ,AT3G25790.1 ,AT5G41740.1 ,AT1G28480.1 ,AT1G74430.1 ,AT3G47220.1 ,AT1G12610.1 ,AT3G01080.1 ,AT5G40350.1 ,AT2G22760.1
GO:0050794	P	regulation of cellular process	27 117 658 31819 2.10E-20 1.50E-18	AT3G28890.1 ,AT4G31800.1 ,AT3G07000.1 ,AT5G43650.1 ,AT1G74080.1 ,AT5G58120.1 ,AT1G01060.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1 ,AT5G67060.1 ,AT3G23250.1 ,AT2G32140.1 ,AT1G28370.1 ,AT4G14370.1 ,AT2G44080.1 ,AT1G01560.1 ,AT4G34410.1 ,AT2G40350.1 ,AT3G25790.1 ,AT5G41740.1 ,AT1G28480.1 ,AT1G74430.1 ,AT3G47220.1 ,AT3G01080.1 ,AT5G40350.1 ,AT2G22760.1
GO:0042221	P	response to chemical stimulus	23 117 439 31819 1.10E-19 6.40E-18	AT4G31800.1 ,AT1G28370.1 ,AT2G34600.1 ,AT1G01060.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1 ,AT4G37220.1 ,AT1G48130.1 ,AT4G13790.1 ,AT3G09940.1 ,AT1G14540.1 ,AT3G23250.1 ,AT2G44080.1 ,AT1G01560.1 ,AT5G52050.1 ,AT4G34410.1 ,AT1G28480.1 ,AT1G74430.1 ,AT5G54610.1 ,AT4G15975.1 ,AT4G21830.1 ,AT5G40350.1
GO:0006950	P	response to stress	23 117 456 31819 2.50E-19 1.20E-17	AT4G31800.1 ,AT1G14540.1 ,AT1G01060.1 ,AT5G58120.1 ,AT1G80840.1 ,AT4G37220.1 ,AT1G48130.1 ,AT3G09940.1 ,AT3G22840.1 ,AT3G23250.1 ,AT2G32140.1 ,AT1G65870.1 ,AT1G80820.1 ,AT2G40350.1 ,AT1G17540.1 ,AT4G14370.1 ,AT1G72520.1 ,AT1G65390.1 ,AT1G12610.1 ,AT4G21830.1 ,AT5G41740.1 ,AT3G13662.1 ,AT3G01080.1
GO:0010468	P	regulation of gene expression	18 117 275 31819 3.70E-17 1.60E-15	AT1G80840.1 ,AT4G31800.1 ,AT1G66725.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1

GO:0045449	P	regulation of transcription	17 117 231 31819 4.70E-17 1.80E-15	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0060255	P	regulation of macromolecule metabolic process	18 117 284 31819 6.30E-17 2.10E-15	AT1G80840.1 ,AT4G31800.1 ,AT1G66725.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0019219	P	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	17 117 239 31819 8.00E-17 2.30E-15	,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0010556	P	regulation of macromolecule biosynthetic process	17 117 238 31819 7.50E-17 2.30E-15	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0051171	P	regulation of nitrogen compound metabolic process	17 117 244 31819 1.10E-16 2.90E-15	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0031326	P	regulation of cellular biosynthetic process	17 117 248 31819 1.40E-16 3.20E-15	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0009889	P	regulation of biosynthetic process	17 117 248 31819 1.40E-16 3.20E-15	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0080090	P	regulation of primary metabolic process 17	17 117 256 31819 2.30E-16 5.00E-15	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0019222	P	regulation of metabolic process 18	18 117 309 31819 2.50E-16 5.10E-15	AT1G80840.1 ,AT4G31800.1 ,AT1G66725.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0006350	P	transcription	17 117 262 31819 3.40E-16 6.30E-15	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0031323	P	regulation of cellular metabolic process	17 117 268 31819 4.80E-16 8.60E-15	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0043170	P	macromolecule metabolic	26 117	AT1G66725.1 ,AT4G31800.1 ,AT5G43650.1 ,AT1G74080.1 ,AT1G01060.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1

		process 26	983 31819 3.40E-15 5.70E-14	,AT1G33770.1 ,AT5G67060.1 ,AT3G23250.1 ,AT4G21323.1 ,AT1G28370.1 ,AT1G61610.1 ,AT4G34410.1 ,AT2G40350.1 ,AT1G17540.1 ,AT3G25790.1 ,AT5G60310.1 ,AT1G74430.1 ,AT1G51820.1 ,AT1G70130.1 ,AT3G59750.1 ,AT3G01080.1 ,AT5G40350.1 ,AT2G22760.1
GO:0044260	P	cellular macromolecule metabolic process	24 117 888 31819 2.90E-14 4.60E-13	AT4G31800.1 ,AT5G43650.1 ,AT1G74080.1 ,AT1G01060.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1 ,AT1G33770.1 ,AT5G67060.1 ,AT3G23250.1 ,AT1G28370.1 ,AT1G61610.1 ,AT4G34410.1 ,AT2G40350.1 ,AT1G17540.1 ,AT3G25790.1 ,AT5G60310.1 ,AT1G74430.1 ,AT1G51820.1 ,AT1G70130.1 ,AT3G59750.1 ,AT3G01080.1 ,AT5G40350.1 ,AT2G22760.1
GO:0006952	P	defense response	11 117 82 31819 4.70E-14 7.20E-13	AT1G65390.1 ,AT1G80840.1 ,AT4G31800.1 ,AT1G65870.1 ,AT4G14370.1 ,AT2G32140.1 ,AT3G01080.1 ,AT5G58120.1 ,AT5G41740.1 ,AT1G72520.1 ,AT3G13662.1
GO:0009058	P	biosynthetic process	23 117 834 31819 7.10E-14 1.00E-12	AT4G31800.1 ,AT5G43650.1 ,AT1G74080.1 ,AT2G35710.3 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1 ,AT5G67060.1 ,AT3G23250.1 ,AT1G28370.1 ,AT1G65870.1 ,AT1G80820.1 ,AT4G34410.1 ,AT3G13662.1 ,AT2G40350.1 ,AT3G25790.1 ,AT1G72520.1 ,AT1G74430.1 ,AT5G28237.1 ,AT1G01060.1 ,AT3G01080.1 ,AT5G40350.1 ,AT2G22760.1
GO:0044249	P	cellular biosynthetic process	22 117 760 31819 1.00E-13 1.50E-12	AT4G31800.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G01080.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1 ,AT5G67060.1 ,AT3G23250.1 ,AT1G28370.1 ,AT1G65870.1 ,AT1G80820.1 ,AT4G34410.1 ,AT3G13662.1 ,AT2G40350.1 ,AT3G25790.1 ,AT1G72520.1 ,AT1G74430.1 ,AT5G28237.1 ,AT1G01060.1 ,AT5G40350.1 ,AT2G22760.1
GO:0009751	P	response to salicylic acid stimulus	6 117 7 31819 3.60E-12 4.80E-11	AT5G54610.1 ,AT4G31800.1 ,AT1G80840.1 ,AT1G01060.1 ,AT1G28480.1 ,AT1G74430.1
GO:0034645	P	cellular macromolecule biosynthetic process	17 117 482 31819 4.30E-12 5.60E-11	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0006139	P	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	18 117 562 31819 4.50E-12 5.60E-11	AT1G80840.1 ,AT4G31800.1 ,AT3G23250.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT4G24340.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0009059	P	macromolecule biosynthetic process	17 117 485 31819 4.70E-12 5.70E-11	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0009743	P	response to carbohydrate stimulus	9 117 62 31819 5.40E-12 6.20E-11	AT1G80840.1 ,AT4G37220.1 ,AT4G31800.1 ,AT4G15975.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G34600.1 ,AT3G23230.1 ,AT2G44840.1
GO:0010467	P	gene expression 18	18 117 575 31819 6.50E-12 7.30E-11	AT1G80840.1 ,AT4G31800.1 ,AT1G66725.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0006807	P	nitrogen compound metabolic process	19 117 702 31819	AT1G80840.1 ,AT4G31800.1 ,AT3G23250.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT5G28237.1 ,AT4G24340.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1

			1.80E-11 2.00E-10	,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0010200	P	response to chitin	8 117 52 31819 5.60E-11 5.90E-10	AT1G80840.1 ,AT4G31800.1 ,AT4G15975.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G34600.1 ,AT3G23230.1 ,AT2G44840.1
GO:0043687	P	post-translational protein modification 7	7 117 36 31819 2.10E-10 2.20E-09	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0016070	P	RNA metabolic process	11 117 232 31819 1.70E-09 1.70E-08	AT1G80840.1 ,AT4G31800.1 ,AT1G28370.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G23230.1 ,AT2G44840.1 ,AT5G40350.1 ,AT1G74430.1
GO:0009723	P	response to ethylene stimulus	6 117 30 31819 3.80E-09 3.70E-08	AT1G28370.1 ,AT3G23250.1 ,AT1G01060.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G74430.1
GO:0006464	P	protein modification process	7 117 144 31819 1.50E-06 1.40E-05	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0009753	P	response to jasmonic acid stimulus	7 117 152 31819 2.10E-06 1.90E-05	AT3G09940.1 ,AT3G23250.1 ,AT2G34600.1 ,AT1G01060.1 ,AT1G28480.1 ,AT5G40350.1 ,AT1G74430.1
GO:0009719	P	response to endogenous stimulus 14	14 117 820 31819 2.40E-06 2.10E-05	AT1G01560.1 ,AT2G44080.1 ,AT1G48130.1 ,AT4G13790.1 ,AT1G28370.1 ,AT3G09940.1 ,AT3G23250.1 ,AT2G34600.1 ,AT1G01060.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G28480.1 ,AT5G40350.1 ,AT1G74430.1
GO:0009628	P	response to abiotic stimulus	9 117 318 31819 3.50E-06 3.00E-05	AT1G48130.1 ,AT4G13790.1 ,AT1G80820.1 ,AT3G09940.1 ,AT1G12610.1 ,AT3G23250.1 ,AT2G40350.1 ,AT1G01060.1 ,AT3G22840.1
GO:0070887	P	cellular response to chemical stimulus	5 117 62 31819 4.90E-06 4.10E-05	AT1G28370.1 ,AT2G44840.1 ,AT1G28480.1 ,AT2G44080.1 ,AT3G23230.1
GO:0043412	P	macromolecule modification	7 117 180 31819 6.10E-06 5.00E-05	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0007165	P	signal transduction	13 117 989 31819 8.10E-05 0.00065	AT1G01560.1 ,AT3G28890.1 ,AT3G07000.1 ,AT2G44080.1 ,AT1G28370.1 ,AT3G23230.1 ,AT3G47220.1 ,AT2G32140.1 ,AT4G14370.1 ,AT5G58120.1 ,AT5G41740.1 ,AT2G44840.1 ,AT1G28480.1

GO:0019538	P	protein metabolic process	8 117 374 31819 8.50E-05 0.00067	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT4G21323.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0006355	P	regulation of transcription, DNA-dependent	11 117 770 31819 0.00014 0.0011	AT1G80840.1 ,AT4G31800.1 ,AT1G28370.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G23230.1 ,AT2G44840.1 ,AT5G40350.1 ,AT1G74430.1
GO:0051252	P	regulation of RNA metabolic process	11 117 775 31819 0.00015 0.0011	AT1G80840.1 ,AT4G31800.1 ,AT1G28370.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G23230.1 ,AT2G44840.1 ,AT5G40350.1 ,AT1G74430.1
GO:0032774	P	RNA biosynthetic process	11 117 812 31819 0.00023 0.0016	AT1G80840.1 ,AT4G31800.1 ,AT1G28370.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G23230.1 ,AT2G44840.1 ,AT5G40350.1 ,AT1G74430.1
GO:0006351	P	transcription, DNA-dependent	11 117 811 31819 0.00022 0.0016	AT1G80840.1 ,AT4G31800.1 ,AT1G28370.1 ,AT4G34410.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G23230.1 ,AT2G44840.1 ,AT5G40350.1 ,AT1G74430.1
GO:0044267	P	cellular protein metabolic process	7 117 338 31819 0.00029 0.002	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0009725	P	response to hormone stimulus	10 117 755 31819 0.00052 0.0036	AT1G01560.1 ,AT2G44080.1 ,AT3G23250.1 ,AT4G13790.1 ,AT1G28370.1 ,AT1G48130.1 ,AT1G01060.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G74430.1
GO:0048856	P	anatomical structure development	6 117 330 31819 0.0015 0.01	AT5G01040.1 ,AT5G67060.1 ,AT1G12610.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1
GO:0007242	P	intracellular signaling cascade 7	7 117 510 31819 0.003 0.02	AT3G07000.1 ,AT2G44080.1 ,AT1G28370.1 ,AT3G47220.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G28480.1
GO:0032501	P	multicellular organismal process	7 117 527 31819 0.0036 0.023	AT1G61610.1 ,AT5G01040.1 ,AT5G67060.1 ,AT1G12610.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1
GO:0006955	P	immune response 5	5 117 282 31819 0.0042 0.027	AT1G80840.1 ,AT2G32140.1 ,AT5G58120.1 ,AT4G31800.1 ,AT5G41740.1
GO:0002376	P	immune system process	5 117 283	AT1G80840.1 ,AT2G32140.1 ,AT5G58120.1 ,AT4G31800.1 ,AT5G41740.1

			31819 0.0042 0.027	
GO:0032502	P	developmental process	7 117 574 31819 0.0057 0.035	AT5G01040.1 ,AT4G30430.1 ,AT5G67060.1 ,AT1G12610.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1
GO:0007275	P	multicellular organismal development	6 117 507 31819 0.012 0.071	AT5G01040.1 ,AT5G67060.1 ,AT1G12610.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1
GO:0009987	P	cellular process	45 117 9400 31819 0.024 0.14	AT1G66725.1 ,AT3G28890.1 ,AT4G31800.1 ,AT3G07000.1 ,AT5G40350.1 ,AT5G43650.1 ,AT1G74080.1 ,AT5G60310.1 ,AT4G24340.1 ,AT1G01060.1 ,AT5G58120.1 ,AT2G44840.1 ,AT1G12610.1 ,AT1G80840.1 ,AT1G28480.1 ,AT5G41080.1 ,AT1G33770.1 ,AT5G67060.1 ,AT1G80820.1 ,AT3G23250.1 ,AT2G32140.1 ,AT3G23230.1 ,AT1G28370.1 ,AT4G14370.1 ,AT1G65870.1 ,AT2G44080.1 ,AT1G01560.1 ,AT5G52050.1 ,AT4G34410.1 ,AT2G40350.1 ,AT1G17540.1 ,AT3G25790.1 ,AT5G41740.1 ,AT1G72520.1 ,AT1G74430.1 ,AT1G51820.1 ,AT1G61610.1 ,AT1G73860.1 ,AT1G70130.1 ,AT3G47220.1 ,AT5G28237.1 ,AT3G59750.1 ,AT3G01080.1 ,AT3G13662.1 ,AT2G22760.1
GO:0006468	P	protein amino acid phosphorylation	7 117 798 31819 0.029 0.17	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0009791	P	post-embryonic development	5 117 520 31819 0.044 0.25	AT5G67060.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1 ,AT5G01040.1
GO:0044238	P	primary metabolic process	35 117 7264 31819 0.047 0.26	AT4G31800.1 ,AT5G40350.1 ,AT5G43650.1 ,AT1G74080.1 ,AT2G43870.1 ,AT4G24340.1 ,AT2G35710.3 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1 ,AT5G41080.1 ,AT1G33770.1 ,AT5G67060.1 ,AT3G23250.1 ,AT4G21323.1 ,AT1G28370.1 ,AT1G65870.1 ,AT1G80820.1 ,AT4G34410.1 ,AT2G40350.1 ,AT1G17540.1 ,AT3G25790.1 ,AT5G60310.1 ,AT1G72520.1 ,AT1G74430.1 ,AT1G51820.1 ,AT1G61610.1 ,AT1G70130.1 ,AT3G47220.1 ,AT5G28237.1 ,AT3G59750.1 ,AT1G01060.1 ,AT3G13662.1 ,AT3G01080.1 ,AT2G22760.1
GO:0016310	P	phosphorylation 7	7 117 897 31819 0.049 0.27	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0006796	P	phosphate metabolic process	7 117 969 31819 0.068 0.37	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0006793	P	phosphorus metabolic process	7 117 970 31819 0.068 0.37	AT1G51820.1 ,AT1G61610.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT5G60310.1
GO:0051716	P	cellular response to stimulus	5 117 625	AT1G28370.1 ,AT2G44840.1 ,AT1G28480.1 ,AT2G44080.1 ,AT3G23230.1

			31819 0.082 0.44	
GO:0008152	P	metabolic process	38 117 8536 31819 0.1 0.54	AT1G66725.1 ,AT4G31800.1 ,AT5G40350.1 ,AT5G43650.1 ,AT1G74080.1 ,AT2G43870.1 ,AT3G46690.1 ,AT4G24340.1 ,AT2G35710.3 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1 ,AT5G41080.1 ,AT1G33770.1 ,AT5G67060.1 ,AT3G23250.1 ,AT4G21323.1 ,AT1G28370.1 ,AT1G65870.1 ,AT1G80820.1 ,AT4G34410.1 ,AT2G40350.1 ,AT1G17540.1 ,AT3G25790.1 ,AT5G60310.1 ,AT1G72520.1 ,AT1G74430.1 ,AT1G51820.1 ,AT1G61610.1 ,AT1G66020.1 ,AT1G70130.1 ,AT3G47220.1 ,AT5G28237.1 ,AT3G59750.1 ,AT1G01060.1 ,AT3G13662.1 ,AT3G01080.1 ,AT2G22760.1
GO:0022414	P	reproductive process	6 117 888 31819 0.11 0.57	AT1G61610.1 ,AT5G01040.1 ,AT5G67060.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1
GO:0000003	P	reproduction	6 117 903 31819 0.12 0.59	AT1G61610.1 ,AT5G01040.1 ,AT5G67060.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1
GO:0048608	P	reproductive structure development	5 117 740 31819 0.14 0.67	AT5G67060.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1 ,AT5G01040.1
GO:0003006	P	reproductive developmental process	5 117 740 31819 0.14 0.67	AT5G67060.1 ,AT1G48130.1 ,AT1G01060.1 ,AT5G40350.1 ,AT5G01040.1
GO:0044237	P	cellular metabolic process	31 117 6970 31819 0.14 0.67	AT4G31800.1 ,AT1G61610.1 ,AT5G43650.1 ,AT1G74080.1 ,AT4G24340.1 ,AT1G01060.1 ,AT3G23230.1 ,AT2G44840.1 ,AT1G80840.1 ,AT5G41080.1 ,AT1G33770.1 ,AT5G67060.1 ,AT3G23250.1 ,AT1G28370.1 ,AT1G65870.1 ,AT1G80820.1 ,AT4G34410.1 ,AT3G13662.1 ,AT2G40350.1 ,AT1G17540.1 ,AT3G25790.1 ,AT5G60310.1 ,AT1G72520.1 ,AT1G74430.1 ,AT1G51820.1 ,AT1G70130.1 ,AT5G28237.1 ,AT3G59750.1 ,AT3G01080.1 ,AT5G40350.1 ,AT2G22760.1
GO:0003700	F	transcription factor activity	18 117 46 31819 1.10E-29 1.10E-27	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT1G12610.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0016740	F	transferase activity	17 117 902 31819 4.40E-08 2.00E-06	AT3G50280.1 ,AT4G21390.1 ,AT3G28890.1 ,AT4G15280.1 ,AT1G51820.1 ,AT1G01560.1 ,AT1G32910.1 ,AT5G60310.1 ,AT1G61610.1 ,AT5G25110.1 ,AT3G46690.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT3G25250.1 ,AT2G35710.3
GO:0016301	F	kinase activity 12	12 117 417 31819 6.40E-08 2.00E-06	AT1G51820.1 ,AT3G28890.1 ,AT1G01560.1 ,AT4G21390.1 ,AT5G60310.1 ,AT1G61610.1 ,AT5G25110.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT3G25250.1
GO:0016772	F	transferase activity, transferring phosphorus-containing groups 12	12 117 447 31819 1.30E-07 3.20E-06	AT1G51820.1 ,AT3G28890.1 ,AT1G01560.1 ,AT4G21390.1 ,AT5G60310.1 ,AT1G61610.1 ,AT5G25110.1 ,AT1G33770.1 ,AT1G70130.1 ,AT1G17540.1 ,AT3G59750.1 ,AT3G25250.1

GO:0046872	F	metal ion binding	7 117 297 31819 0.00013 0.0019	AT3G18590.1 ,AT3G07000.1 ,AT1G67856.1 ,AT1G66020.1 ,AT1G14540.1 ,AT4G15975.1 ,AT1G72520.1
GO:0043169	F	cation binding	7 117 306 31819 0.00016 0.0019	AT3G18590.1 ,AT3G07000.1 ,AT1G67856.1 ,AT1G66020.1 ,AT1G14540.1 ,AT4G15975.1 ,AT1G72520.1
GO:0043167	F	ion binding	7 117 306 31819 0.00016 0.0019	AT3G18590.1 ,AT3G07000.1 ,AT1G67856.1 ,AT1G66020.1 ,AT1G14540.1 ,AT4G15975.1 ,AT1G72520.1
GO:0030528	F	transcription regulator activity	18 117 1869 31819 0.00017 0.0019	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT1G12610.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0046914	F	transition metal ion binding	6 117 195 31819 0.0001 0.0019	AT3G18590.1 ,AT3G07000.1 ,AT1G67856.1 ,AT4G15975.1 ,AT1G14540.1 ,AT1G72520.1
GO:0016491	F	oxidoreductase activity 9	9 117 555 31819 0.00024 0.0023	AT5G01040.1 ,AT1G48130.1 ,AT5G44440.1 ,AT1G80820.1 ,AT3G09940.1 ,AT1G14540.1 ,AT4G21830.1 ,AT1G72520.1 ,AT2G27690.1
GO:0005515	F	protein binding 11	11 117 955 31819 0.00086 0.0075	AT5G54610.1 ,AT4G11000.1 ,AT2G24600.1 ,AT3G07000.1 ,AT4G15975.1 ,AT5G57010.1 ,AT1G67856.1 ,AT3G28890.1 ,AT4G21323.1 ,AT5G58120.1 ,AT5G41740.1
GO:0003677	F	DNA binding	18 117 2229 31819 0.0014 0.011	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT1G12610.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0005488	F	binding 45	45 117 9111 31819 0.014 0.1	AT1G56240.1 ,AT1G33770.1 ,AT3G28890.1 ,AT4G31800.1 ,AT3G07000.1 ,AT1G67856.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT1G01060.1 ,AT5G58120.1 ,AT2G44840.1 ,AT1G12610.1 ,AT2G27690.1 ,AT1G80840.1 ,AT5G52400.1 ,AT3G23250.1 ,AT5G67060.1 ,AT3G22840.1 ,AT1G14540.1 ,AT4G21323.1 ,AT1G28370.1 ,AT4G14370.1 ,AT3G18590.1 ,AT4G21390.1 ,AT4G11000.1 ,AT5G44440.1 ,AT4G34410.1 ,AT2G40350.1 ,AT3G25790.1 ,AT5G41740.1 ,AT1G72520.1 ,AT1G74430.1 ,AT1G65390.1 ,AT5G54610.1 ,AT1G61610.1 ,AT1G73860.1 ,AT2G24600.1 ,AT4G15975.1 ,AT5G57010.1 ,AT1G66020.1 ,AT5G28237.1 ,AT3G01080.1 ,AT5G40350.1 ,AT2G22760.1
GO:0003824	F	catalytic activity	39 117 7726 31819 0.017 0.12	AT1G33770.1 ,AT3G28890.1 ,AT5G01040.1 ,AT1G61610.1 ,AT2G43870.1 ,AT3G46690.1 ,AT4G24340.1 ,AT2G35710.3 ,AT5G58120.1 ,AT2G27690.1 ,AT1G01560.1 ,AT5G41080.1 ,AT1G48130.1 ,AT4G30280.1 ,AT1G32910.1 ,AT2G47050.1 ,AT3G09940.1 ,AT5G25110.1 ,AT1G14540.1 ,AT4G21323.1 ,AT3G25250.1 ,AT4G21390.1 ,AT4G15280.1 ,AT1G80820.1 ,AT5G44440.1 ,AT4G25810.1 ,AT1G70130.1 ,AT1G17540.1 ,AT5G60310.1 ,AT1G72520.1 ,AT3G50280.1 ,AT1G51820.1 ,AT1G73860.1 ,AT1G66020.1 ,AT3G47220.1 ,AT5G28237.1

				,AT3G59750.1 ,AT4G21830.1 ,AT5G41740.1
GO:0030554	F	adenyl nucleotide binding	6 117 645 31819 0.033 0.18	AT1G61610.1 ,AT5G44440.1 ,AT5G58120.1 ,AT1G33770.1 ,AT1G73860.1 ,AT5G41740.1
GO:0001883	F	purine nucleoside binding	6 117 645 31819 0.033 0.18	AT1G61610.1 ,AT5G44440.1 ,AT5G58120.1 ,AT1G33770.1 ,AT1G73860.1 ,AT5G41740.1
GO:0001882	F	nucleoside binding	6 117 645 31819 0.033 0.18	AT1G61610.1 ,AT5G44440.1 ,AT5G58120.1 ,AT1G33770.1 ,AT1G73860.1 ,AT5G41740.1
GO:0017076	F	purine nucleotide binding	6 117 822 31819 0.084 0.43	AT1G61610.1 ,AT5G44440.1 ,AT5G58120.1 ,AT1G33770.1 ,AT1G73860.1 ,AT5G41740.1
GO:0005524	F	ATP binding	5 117 645 31819 0.091 0.43	AT1G33770.1 ,AT5G41740.1 ,AT1G61610.1 ,AT1G73860.1 ,AT5G58120.1
GO:0032559	F	adenyl ribonucleotide binding	5 117 645 31819 0.091 0.43	AT1G33770.1 ,AT5G41740.1 ,AT1G61610.1 ,AT1G73860.1 ,AT5G58120.1
GO:0003676	F	nucleic acid binding	18 117 3868 31819 0.18 0.78	AT1G80840.1 ,AT4G31800.1 ,AT5G67060.1 ,AT5G43650.1 ,AT1G74080.1 ,AT3G23230.1 ,AT4G34410.1 ,AT1G12610.1 ,AT3G23250.1 ,AT2G40350.1 ,AT3G01080.1 ,AT3G25790.1 ,AT1G28370.1 ,AT1G01060.1 ,AT2G44840.1 ,AT2G22760.1 ,AT5G40350.1 ,AT1G74430.1
GO:0032555	F	purine ribonucleotide binding	5 117 822 31819 0.19 0.78	AT1G33770.1 ,AT5G41740.1 ,AT1G61610.1 ,AT1G73860.1 ,AT5G58120.1
GO:0032553	F	ribonucleotide binding	5 117 822 31819 0.19 0.78	AT1G33770.1 ,AT5G41740.1 ,AT1G61610.1 ,AT1G73860.1 ,AT5G58120.1
GO:0000166	F	nucleotide binding	6 117 1851 31819 0.68 1	AT1G61610.1 ,AT5G44440.1 ,AT5G58120.1 ,AT1G33770.1 ,AT1G73860.1 ,AT5G41740.1
GO:0016787	F	hydrolase activity	10 117 2819 31819 0.59 1	AT1G73860.1 ,AT5G41080.1 ,AT4G30280.1 ,AT4G25810.1 ,AT2G47050.1 ,AT3G47220.1 ,AT4G21323.1 ,AT2G43870.1 ,AT5G58120.1 ,AT5G41740.1
GO:0005634	C	nucleus 9	9	AT1G80840.1 ,AT4G31800.1 ,AT5G43650.1 ,AT3G23230.1

			117 212 31819 1.30E-07 7.80E-06	,AT4G34410.1 ,AT2G40350.1 ,AT1G28370.1 ,AT2G44840.1 ,AT2G22760.1
GO:0044464	C	cell part	45 117 6952 31819 3.60E-05 0.0007	AT4G31800.1 ,AT1G61610.1 ,AT5G43650.1 ,AT3G23230.1 ,AT5G53710.1 ,AT2G43870.1 ,AT4G24340.1 ,AT2G35710.3 ,AT5G58120.1 ,AT2G44840.1 ,AT2G27690.1 ,AT2G22880.1 ,AT4G27654.1 ,AT1G80840.1 ,AT3G43850.1 ,AT1G02700.1 ,AT2G47050.1 ,AT3G09940.1 ,AT1G14540.1 ,AT2G32140.1 ,AT4G21323.1 ,AT1G28370.1 ,AT3G18590.1 ,AT1G65870.1 ,AT5G52050.1 ,AT2G44080.1 ,AT5G44440.1 ,AT4G34410.1 ,AT2G40350.1 ,AT4G14370.1 ,AT5G60310.1 ,AT1G72520.1 ,AT5G38700.1 ,AT1G51820.1 ,AT1G73860.1 ,AT4G15975.1 ,AT1G70130.1 ,AT3G59750.1 ,AT5G14110.1 ,AT4G27657.1 ,AT4G21830.1 ,AT5G41740.1 ,AT2G22760.1 ,AT3G13662.1 ,AT1G35140.1
GO:0005623	C	cell	45 117 6952 31819 3.60E-05 0.0007	AT4G31800.1 ,AT1G61610.1 ,AT5G43650.1 ,AT3G23230.1 ,AT5G53710.1 ,AT2G43870.1 ,AT4G24340.1 ,AT2G35710.3 ,AT5G58120.1 ,AT2G44840.1 ,AT2G27690.1 ,AT2G22880.1 ,AT4G27654.1 ,AT1G80840.1 ,AT3G43850.1 ,AT1G02700.1 ,AT2G47050.1 ,AT3G09940.1 ,AT1G14540.1 ,AT2G32140.1 ,AT4G21323.1 ,AT1G28370.1 ,AT3G18590.1 ,AT1G65870.1 ,AT5G52050.1 ,AT2G44080.1 ,AT5G44440.1 ,AT4G34410.1 ,AT2G40350.1 ,AT4G14370.1 ,AT5G60310.1 ,AT1G72520.1 ,AT5G38700.1 ,AT1G51820.1 ,AT1G73860.1 ,AT4G15975.1 ,AT1G70130.1 ,AT3G59750.1 ,AT5G14110.1 ,AT4G27657.1 ,AT4G21830.1 ,AT5G41740.1 ,AT2G22760.1 ,AT3G13662.1 ,AT1G35140.1
GO:0044425	C	membrane part	6 117 364 31819 0.0025 0.036	AT3G18590.1 ,AT5G58120.1 ,AT2G32140.1 ,AT5G14110.1 ,AT4G14370.1 ,AT5G41740.1
GO:0031224	C	intrinsic to membrane	6 117 680 31819 0.041 0.48	AT3G18590.1 ,AT5G58120.1 ,AT2G32140.1 ,AT5G14110.1 ,AT4G14370.1 ,AT5G41740.1
GO:0012505	C	endomembrane system	17 117 3152 31819 0.07 0.69	AT1G51820.1 ,AT1G61610.1 ,AT4G27657.1 ,AT4G15975.1 ,AT1G14540.1 ,AT2G47050.1 ,AT5G53710.1 ,AT2G43870.1 ,AT4G24340.1 ,AT1G70130.1 ,AT4G21323.1 ,AT2G35710.3 ,AT3G59750.1 ,AT4G27654.1 ,AT5G60310.1 ,AT3G13662.1 ,AT5G44440.1
GO:0016020	C	membrane	7 117 3495 31819 0.98 1	AT3G18590.1 ,AT5G52050.1 ,AT5G58120.1 ,AT2G32140.1 ,AT5G14110.1 ,AT4G14370.1 ,AT5G41740.1
GO:0043231	C	intracellular membrane-bounded organelle	17 117 6132 31819 0.93 1	AT1G80840.1 ,AT1G73860.1 ,AT4G31800.1 ,AT5G43650.1 ,AT3G23230.1 ,AT4G34410.1 ,AT2G32140.1 ,AT2G22880.1 ,AT3G43850.1 ,AT2G40350.1 ,AT1G28370.1 ,AT5G41740.1 ,AT2G44840.1 ,AT1G72520.1 ,AT1G02700.1 ,AT5G38700.1 ,AT2G22760.1
GO:0044424	C	intracellular part	19 117 7433 31819 0.98 1	AT1G80840.1 ,AT1G73860.1 ,AT4G31800.1 ,AT5G43650.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G09940.1 ,AT2G22880.1 ,AT3G43850.1 ,AT2G40350.1 ,AT1G28370.1 ,AT4G21830.1 ,AT5G41740.1 ,AT2G44840.1 ,AT1G72520.1 ,AT1G02700.1 ,AT5G38700.1 ,AT2G32140.1 ,AT2G22760.1
GO:0043229	C	intracellular organelle 17	117 6542 31819 0.96	AT1G80840.1 ,AT1G73860.1 ,AT4G31800.1 ,AT5G43650.1 ,AT3G23230.1 ,AT4G34410.1 ,AT2G32140.1 ,AT2G22880.1 ,AT3G43850.1 ,AT2G40350.1 ,AT1G28370.1 ,AT5G41740.1 ,AT2G44840.1 ,AT1G72520.1 ,AT1G02700.1 ,AT5G38700.1

			1	,AT2G22760.1
GO:0043227	C	membrane-bounded organelle	17 117 6138 31819 0.93 1	AT1G80840.1 ,AT1G73860.1 ,AT4G31800.1 ,AT5G43650.1 ,AT3G23230.1 ,AT4G34410.1 ,AT2G32140.1 ,AT2G22880.1 ,AT3G43850.1 ,AT2G40350.1 ,AT1G28370.1 ,AT5G41740.1 ,AT2G44840.1 ,AT1G72520.1 ,AT1G02700.1 ,AT5G38700.1 ,AT2G22760.1
GO:0044444	C	cytoplasmic part	10 117 5113 31819 0.99 1	AT3G09940.1 ,AT3G43850.1 ,AT2G32140.1 ,AT1G73860.1 ,AT4G21830.1 ,AT5G41740.1 ,AT2G22880.1 ,AT1G72520.1 ,AT1G02700.1 ,AT5G38700.1
GO:0009536	C	plastid 5	117 2507 31819 0.96 1	AT5G41740.1 ,AT2G22880.1 ,AT1G72520.1 ,AT2G32140.1 ,AT1G73860.1
GO:0043226	C	organelle	17 117 6546 31819 0.96 1	AT1G80840.1 ,AT1G73860.1 ,AT4G31800.1 ,AT5G43650.1 ,AT3G23230.1 ,AT4G34410.1 ,AT2G32140.1 ,AT2G22880.1 ,AT3G43850.1 ,AT2G40350.1 ,AT1G28370.1 ,AT5G41740.1 ,AT2G44840.1 ,AT1G72520.1 ,AT1G02700.1 ,AT5G38700.1 ,AT2G22760.1
GO:0005622	C	intracellular	20 117 7736 31819 0.98 1	AT1G80840.1 ,AT1G73860.1 ,AT4G31800.1 ,AT2G44080.1 ,AT5G43650.1 ,AT3G23230.1 ,AT4G34410.1 ,AT3G09940.1 ,AT2G22880.1 ,AT3G43850.1 ,AT2G40350.1 ,AT1G28370.1 ,AT4G21830.1 ,AT5G41740.1 ,AT2G44840.1 ,AT1G72520.1 ,AT1G02700.1 ,AT5G38700.1 ,AT2G32140.1 ,AT2G22760.1
GO:0009507	C	chloroplast	5 117 2335 31819 0.94 1	AT5G41740.1 ,AT2G22880.1 ,AT1G72520.1 ,AT2G32140.1 ,AT1G73860.1
GO:0005737	C	cytoplasm	10 117 5501 31819 1 1	AT3G09940.1 ,AT3G43850.1 ,AT2G32140.1 ,AT1G73860.1 ,AT4G21830.1 ,AT5G41740.1 ,AT2G22880.1 ,AT1G72520.1 ,AT1G02700.1 ,AT5G38700.1

CHAPTER 6.

Strain specific interaction between *Aspergillus versicolor* and plants through volatile organic compounds

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6.1 Introduction

Fungi in the genus *Aspergillus* are ubiquitous in terrestrial ecosystems and their spores are common in soil and air. Several species are biotechnologically important as producers of industrial metabolites such as citric acid and lovastatin, while yet others have negative impact in food spoilage and mycotoxin contamination of agricultural products (Bennett and Klich, 1992; Perrone et al., 2007; Machida and Gomi, 2010). *A. fumigatus*, *A. flavus*, and other members of the genus that can grow at 37 ^{o}C are opportunistic human pathogens that can cause life threatening systemic infections (Latge, 1999; Goldman and Osmani, 2008). *A. versicolor*, *A. fumigatus*, *A. flavus*, and *A. terreus* are commonly found in urban ecosystems and their presence is considered an indicator for moisture problems in buildings (Samson et al., 1994; Pasanen et al., 1997; Fomicheva et al., 2006).

For several decades, the volatile organic compounds (VOCs) emitted by molds, including members of the genus *Aspergillus*, have been used as indirect biomarkers to detect fungi in the food supply, the built environment, and human disease (Borjesson et al., 1989; Wessen et al., 2001; Chambers et al., 2009; Heddergott et al., 2014; Sun et al., 2014). Compounds such as 1-octen-3-ol, 3-methyl-1-butanol, and 2-methyl-1-propanol are often produced in large quantities by *Aspergillus* species (Pasanen et al., 1997; Gao et al., 2002; Jurjevic et al., 2008). For Aspergilli and for other molds, the volatile profile is dependent on multiple parameters such as geography, time, and environmental conditions including nutrient content, microbial community composition, temperature, humidity, and pH (McNeal and Herbert, 2009; Insam and Seewald, 2010). Moreover different isolates and strains of a given species may display different volatile profiles. Most volatile

analysis has been focused on its use as an indirect marker to detect the presence of harmful *Aspergillus* species (Schleibinger et al., 2005; Roze et al., 2007; Jurjevic et al., 2008; Chippendale et al., 2014; Heddergott et al., 2014).

Because *Aspergillus versicolor* produces sterigmatocystin, a mycotoxin that damages the liver and kidney of experimental animals, it is considered a potential health risk (Cole and Cox, 1981). Sterigmatocystin is classified as a class II carcinogen by the International Agency for Research on Cancer (IARC, 1987). *A. versicolor* is commonly found in soil environments, plant material, and food. Moreover, as one of the early colonizers of moist surfaces, it is often the most common species found in damp indoor environments (Hodgson et al., 1998; Engelhart et al., 2002). For example, *A versicolor* was the most common species isolated from fiberglass duct liners in houses whose occupants complained of unacceptable moldy odors (Ezeonu et al., 1994) It produces over 40 VOCs comprised of alcohols, aldehydes, aromatics, esters, heterocycles, ketones, terpenes, and so forth (Pasanen et al., 1997).

In recent years, increasing numbers of reports demonstrate that low concentrations of certain fungal VOCs can have potent physiological effects within and between species where they function in signaling, communication, antagonism, and other inter-organism interactions (Korpi et al., 2009; Bennett et al., 2012; Hung et al., 2015). In particular, microbes whose natural habitat is the rhizosphere have been shown to influence plant growth in various ways (Insam and Seewald, 2010; Blom et al., 2011; Bitas et al., 2013).

Our laboratory has used *Arabidopsis thaliana* to study the physiological effects of fungal VOCs (Hung et al., 2013; 2015; Lee et al., 2015). We hypothesized that the

VOCs of *A. versicolor*, an organism whose natural habitat is soil, might affect plant growth. Our study had three goals. First, we sought to detect strain differences in the VOC profiles of two isolates of *A. versicolor* using SPME/GC-MS. The second goal was to determine if the mixtures of VOCs emitted by growing cultures of the two *A. versicolor* strains could mediate changes in growth and seed germination in the plant model, *Arabidopsis thaliana*. Our third goal was to identify individual bioactive VOCs from the VOC mixture emitted by the two *A. versicolor* strains.

6.2 Materials and methods

Aspergillus strains and growth conditions

Aspergillus versicolor strains SRRC 108 (NRRL 3449) and SRRC 2559 (ATCC 32662) were obtained from Dr. Geromy G. Moore at USDA-ARS-SRRC, New Orleans, LA. SRRC 2559 had come to our attention because it caused a participant in a taxonomic workshop to experience an unpleasant itching, while *A. versicolor* strain SRRC 108 did not cause this effect.

For volatile analysis, an aqueous spore suspension was made from a two-week old colony grown on potato dextrose agar. Five ml of substrates, yeast extract sucrose agar (YES) media, water saturated gypsum wallboard (WB), or water saturated ceiling tiles (CT) were placed into a 10 ml vial. YES was inoculated using a 50 µl spore suspension every two days for the duration of 14 days while building materials CT and WB were inoculated on the first and eighth day. At the end of the 14 days of fungal growth, all vials were sealed for 24 hours to build up VOCs in the headspace to be analyzed. Negative controls were fungal free substrates. For the plant exposure experiment, the fungi were grown in 35 × 10 mm Petri dishes on four ml of YES media, and incubated

for five days at 27 ± 1 °C in high humidity prior to the start of the volatile exposure experiments.

Arabidopsis growth conditions

Arabidopsis thaliana seeds (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. Surface-sterilized seeds were sown onto a 100 × 15 mm partitioned Petri dish (split or I-plate) or 60 x 15 mm Petri dish containing Murashige and Skoog medium with vitamins, 3 % sucrose, and 0.03 % phytagel (pH 5.7) (Phytotechnology Laboratories, KS). Seeds were stratified at 4 °C for 3 days prior to volatile exposure.

VOC analysis by headspace SPME/GC-MS

Solid phase microextraction (SPME) was employed as follows: a 1 cm fiber of Carboxen/DVM/PDMS (Supelco, Inc. Bellefonte, PA), stationary phase, was inserted into the headspace and exposed for 15 minutes. Samples were heated to 65 ˚C for adsorption and injected to GC and desorbed at 270 ˚C for 1 minute. After removal, the fiber was heated at 270 ˚C for 2.5 minutes. For analysis, a Combi-Pal autosampler (Leap Technologies, Carrboro, NC) was used with an Agilent 6890 GC (Agilent Inc., Palo Alto, CA) equipped with a 30 m DB-5 column with a 0.25 mm internal diameter, 1.0 µm film thickness, and phase thickness 5% of cross-linked phenylmethylsilicate. Helium carrier gas was injected at 40 cm/sec at 25 psi, while the column temperature was ramped up from 50 ˚C for 1 minute, to 100 ˚C at 5 ˚C/min, and then to 200 ˚C at 10 ˚C/min, then to 270 ˚C at 25 ˚C/min and held for 3 minutes for detection. Agilent 5973 MSD scan mode from m/z 40 to 300 m/z; employing electron ionization.

Compounds were initially identified by library match (Wiley Registry of Mass Spectral Data 7th edition with NIST98 Spectra, Palisdae Corporation, Newfield, NY). Reference standards and extracts were analyzed under the same conditions to confirm the library identification. Specific compounds such as 1-octen-3-ol, 3-methyl-1-butanol, hexanal, and 3-methyl-furan were integrated on selected ions indicative of the compound. The chromatographic traces were compared to identify different headspace compositions and the identification of signature compounds.

Plant exposure to VOCs of Aspergillus versicolor

A double plate-within-a-plate system was used for plant exposure to *Aspergillus* VOCs according to previously described methods (Lee et al., 2015). A small Petri plate (35 × 10 mm) containing *Aspergillus* grown on YES was placed into a larger partitioned Petri dish (100 x 15 mm) containing five stratified *A. thaliana* seeds. Plants and fungi were grown together in a growth chamber at 23 ± 1 °C with a 16-hour photoperiod for 14 days. For controls, plants were grown without exposure to fungi, i.e. the smaller Petri dish contained only medium. At the end of the exposure period, the plants were removed from the exposure conditions and photographed. Then the shoots were separated from the roots and weighed to obtain a fresh weight before the total chlorophyll concentration was determined.

Plant exposure to chemical standards of individual VOCs

The major compounds identified through SPME analysis were selected for further study. Chemical standards of 2-methyl-1-butanol, 3-methyl-1-butanol, 1-octen-3-ol, limonene, and β-farnesene were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). For comparison, we also included β-caryophyllene, a natural bicyclic

sesquiterpene. We exposed seeds and plants to a 0.5 µl/l (vol/vol) concentration of each individual compound. These germination assays and vegetative exposure assays were performed with minor modification as described previously (Lee et al., 2014).

A Petri dish containing 50 surface sterilized *A. thaliana* seeds was placed into a glass tissue culture jar. An appropriate aliquot of each compound was added, volatilized, and the jar was sealed with a translucent polypropylene screw cap. The seeds were exposed to the compound for 72 hours in a growth chamber at 23 $\pm$ 1 ˚C with a 16-hour photoperiod. The control seeds were exposed to the same conditions without the addition of VOCs. At the end of the exposure, the seeds were removed and examined visually using light microscopy. The seeds were scored into three categories, no germination, germinated (presence of radical), and seedling formation (presence of radical, hypocotyls, and cotyledons). For the vegetative exposure assay, five plants were grown in a growth chamber at 23 $\pm$ 1 ˚C with a 16-hour photoperiod for 14 days following stratification. A Petri plate containing 14-day-old plants was placed into a glass tissue culture jar and then the aliquot of each respective compound was added, volatilized, the jar was sealed, and then placed in the growth chamber for 72 hours. At the end of the 72-hour exposure, the plants were removed from experimental conditions and observed for leaf size, color, and other morphological features. The individual plants were then weighed and assayed for total chlorophyll concentration.

Chlorophyll quantification

Total chlorophyll concentration of plants exposed to *Trichoderma* VOCs was determined by submerging the shoot overnight in 1 ml of 80 % acetone in the dark at 4 °C. The total chlorophyll concentration (chlorophyll a and b) was calculated from the

equation [(8.02)(A663) + (20.2)(A645)]*V*/1000 *W*, where *V* is volume and *W* is plant fresh weight. The chlorophyll data were expressed in relation to the fresh weight of the plant shoot.

Three replicates were used per treatment condition, and the experiments were repeated three times. Quantitative results were expressed as standard error of the mean and analyzed using Excel software (Microsoft, Redmond, WA) and SigmaPlot (SPSS Science Inc., IL). Student's t-test and/or one-way analysis of variance (ANOVA) between groups were performed for all quantitative data.

6.3 Results

Volatile analysis

The volatile compounds were identified from headspace analysis of *A. versicolor* strains SRRC 2559 and SRRC 108 grown on YES media, ceiling tile, or wall-board (Table 1). Both strains grew best on YES, where SRRC 2559 emitted 14 compounds and SRRC 108 emitted 12 compounds. The 14 VOCs detected by SPME analysis included three alcohols, one ketone, one alkene, two heterocyclic aromatics, and seven terpenes and terpene derivatives. The two unidentified diterpenes were found only from strain 2559 on YES and ceiling tile. With the exception of limonene, strain 108 did not produce any detectable terpenes on either ceiling tile or wall board. Five VOCs (2-methyl-1-butanol, 3-methyl-butanol, 1-octen-3-ol, dimethoxy benzene and limonene) were produced by both strains on all three substrates. However, relative volatile concentrations varied with the substrate. For both strains, the dominant peak observed on ceiling tile and wall-board was 1-octen-3-ol; whereas 2-methyl-1-butanol, 3-methyl-butanol, and limonene were observed at only trace levels on these substrates.

The amount of each compound produced changed over time. On YES, the highest levels of 3-methyl-1-butanol were observed at Day 4 and steadily decreased through Day 14 (Fig. 1). Similar results were observed for 2-methyl-1-butanol, 3-hexanone, and 1-octen-3-ol (data not shown). The emission of terpenes, sesquiterpenes and diterpenes varied most with time. We observed a steady production of limonene in *A. versicolor* SRRC 2559 while SRRC 108 exhibited an initial increase followed by a steady decline after five days of growth (Fig. 2). Similarly, β-farnesene {7,11-dimethyl-3-methylene-dodeca-1,(E)6,10-triene} increased over time in SRRC 2559 and decreased in SRRC 108. Two diterpenes were produced exclusively by SRRC 2559 (Fig. 3).

		A. versicolor SRRC 2559			*A. versicolor* SRRC 108		
Compound	**RT**	**YES**	**CT**	**WB**	**YES**	**CT**	**WB**
Alcohols							
2-methyl-1-butanol	6.64	+	+	+	+	+	+
3-methyl-1-butanol	6.52	+	+	+	+	+	+
1-octen-3-ol	14.06	+	+	+	+	+	+
Ketone							
3-hexanone	8.11	+	—	—	+	—	—
Alkene							
(5Z)-octa-1,5-dien-3-ol	13.96	+	+	—	+	—	—
Heterocyclic aromatic							
tetramethyl-pyrazine	16.60	+	—	—	+	—	—
dimethoxy benzene	18.18	+	+	+	+	+	+
Terpenes and terpene derivatives							
limonene	15.47	+	+	+	+	+	+
terpinolelene	18.69	+	+	—	+	—	—
2-norpinene	22.28	+	+	—	+	—	—
β-farnesene	22.82	+	+	—	+	—	—
sesquiterpene 2	22.93	+	+	—	+	—	—
diterpene 1	26.18	+	+	—	—	—	—
diterpene 2	26.66	+	+	—	—	—	—

Table 1. Headspace analysis (SPME/GC-MS) of volatiles collected from *Aspergillus versicolor* SRRC 2559 and SRRC 108 grown on yeast extract sucrose agar (YES) media, ceiling tile (CT), and wallboard (WB). Retention time (RT) is given in minutes. + denotes presence of compound and – denotes none detected.

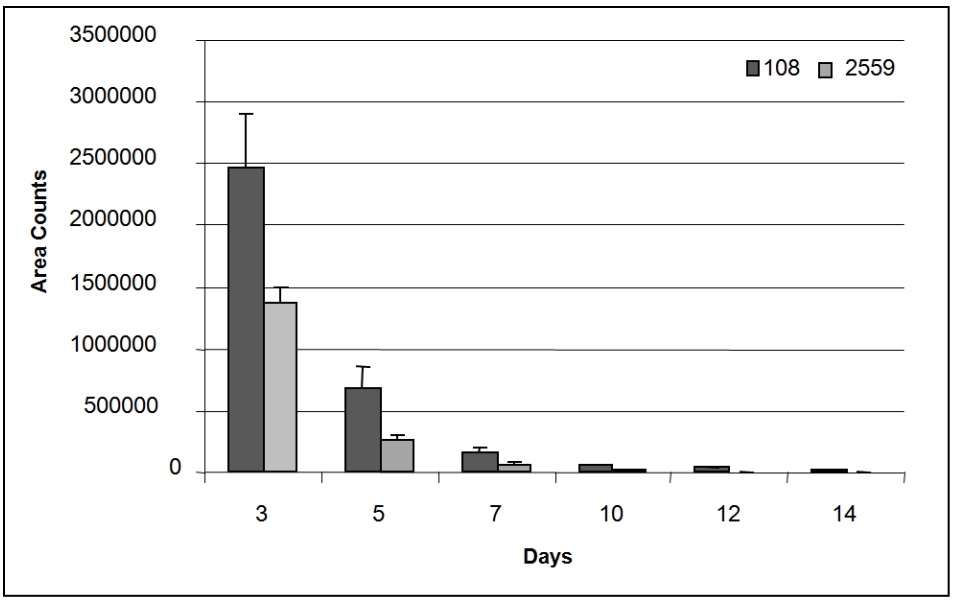

Figure 1. Comparison of 3-methyl-1-butanol production from *Aspergillus versicolor* SRRC 2559 and SRRC 108 grown on yeast extract sucrose (YES) media from Day 3 - 14. * denotes significance.

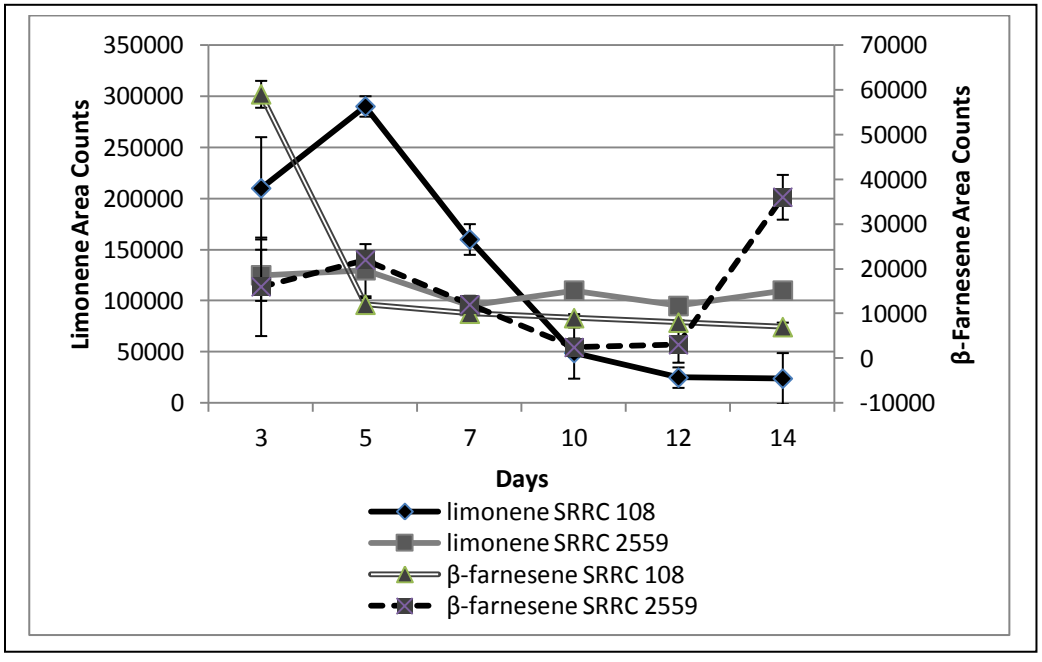

Figure 2. Production of limonene and β-farnesene from *Aspergillus versicolor* SRRC 2559 and SRRC 108 grown on YES media over 14-day observation period.

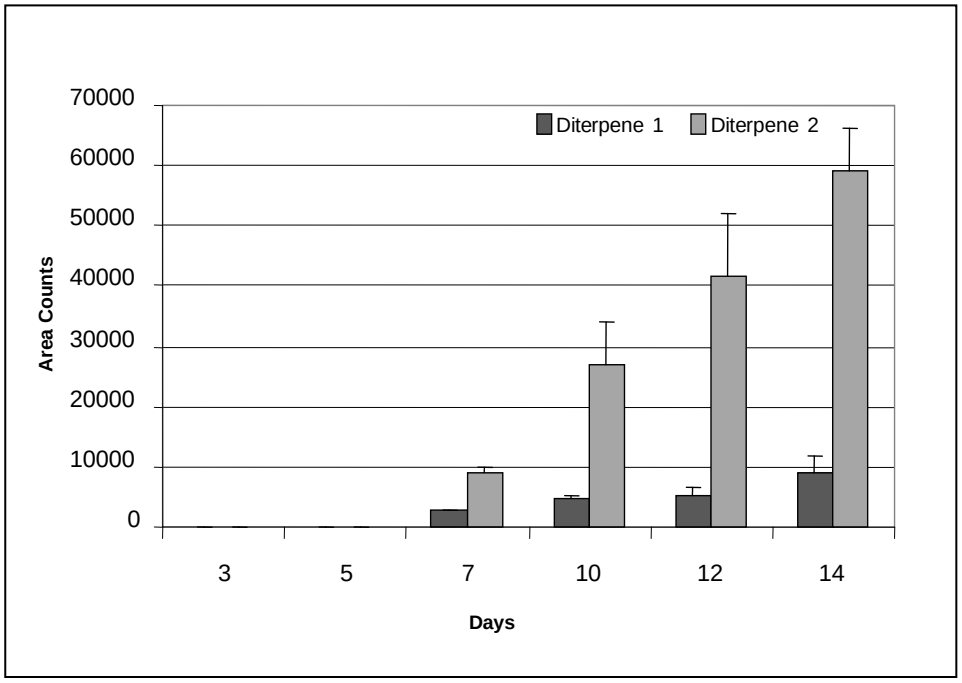

Figure 3. Production of diterpenes by *Aspergillus versicolor* SRRC 2559 grown on YES media over 14-day observation period.

Differential plant responses to VOCs from growing cultures of A. versicolor

Arabidopsis thaliana seeds were grown together with *A. versicolor* SRRC 2559 or SRRC 108 for 14 days. The split plate assay (Fig. 4) provides physical separation while allowing gas exchange to occur between the organisms. In general, plants exposed to VOCs emitted by SRRC 2559 exhibited reduction in plant size, fewer leaves, and decrease in root growth (Fig. 4A and 4B). Plants exposed to SRRC 108 were similar to control plants both in size and number of leaves present. These plant growth and developmental stages were in accordance with established phenotypic characteristics for *A. thaliana* (Boyes et al., 2001). However, compared to controls, we saw a reduction in root growth among plants exposed to SRRC 108. We also observed accumulation of reddish-purple pigment in the underside of plant leaves (Fig. 4B). Although pigment was

observed in plants exposed to both SRRC 2559 and SRRC 108, more plants exposed to a shared atmosphere with VOCs from SRRC 2559 displayed pigment production.

Average shoot fresh weight and total chlorophyll concentration of plants exposed to naturally occurring mixtures of *A. versicolor* volatiles are plotted in Figure 5. Plants were exposed to VOCs emitted by SRRC 259 and SRRC 108 grown on YES media for 14 days. The average chlorophyll concentration of control plants was 0.76 $\pm$ 0.13 mg per gram of fresh tissue with a total shoot weight of 25 $\pm$ 2 mg. Although plants exposed to SRRC 2559 had comparable shoot fresh weight (0.24 $\pm$ 3 mg), there was a significance reduction in total chlorophyll, a 51% decrease. Plants exposed to SRRC 108 had slightly higher fresh weight while the total chlorophyll did not differ significantly compared to controls (ANOVA, $P = 0.01$).

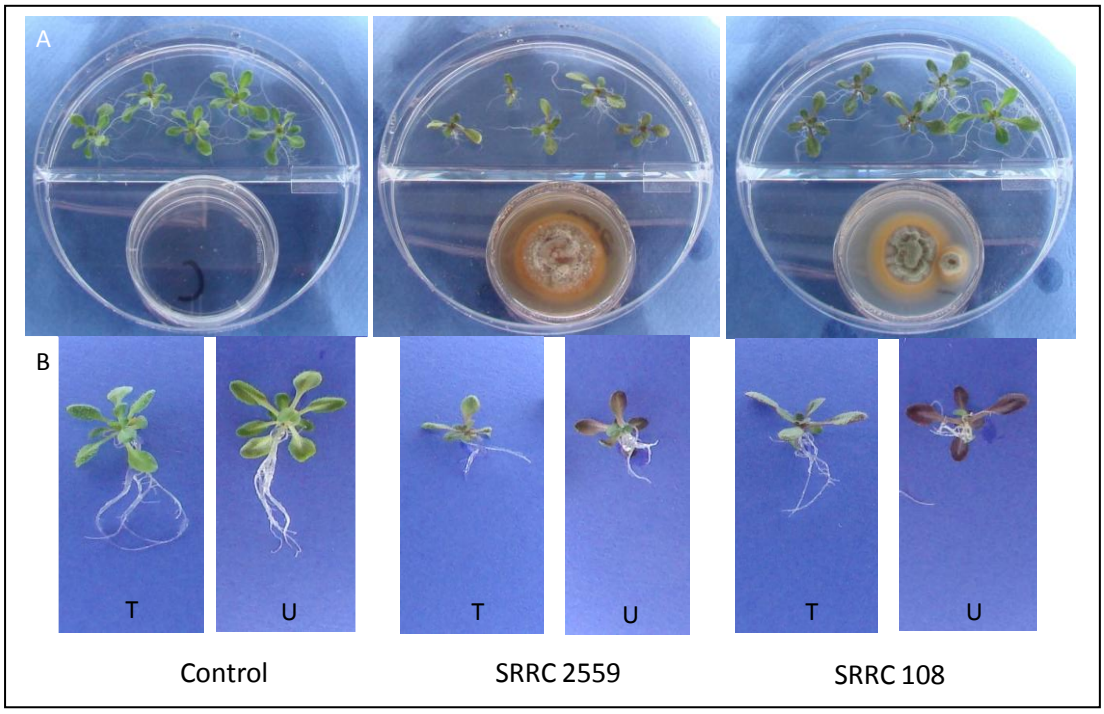

Figure 4. *Arabidopsis thaliana* exposed to *Aspergillus versicolor* SRRC 2559 and SRRC 108 for 14 days. (A) Split plates showing controls and *A. versicolor* exposed plants. (B) *Arabidopsis* plants removed from growth medium. Top (T) and underside (U) of plants exposed to VOCs.

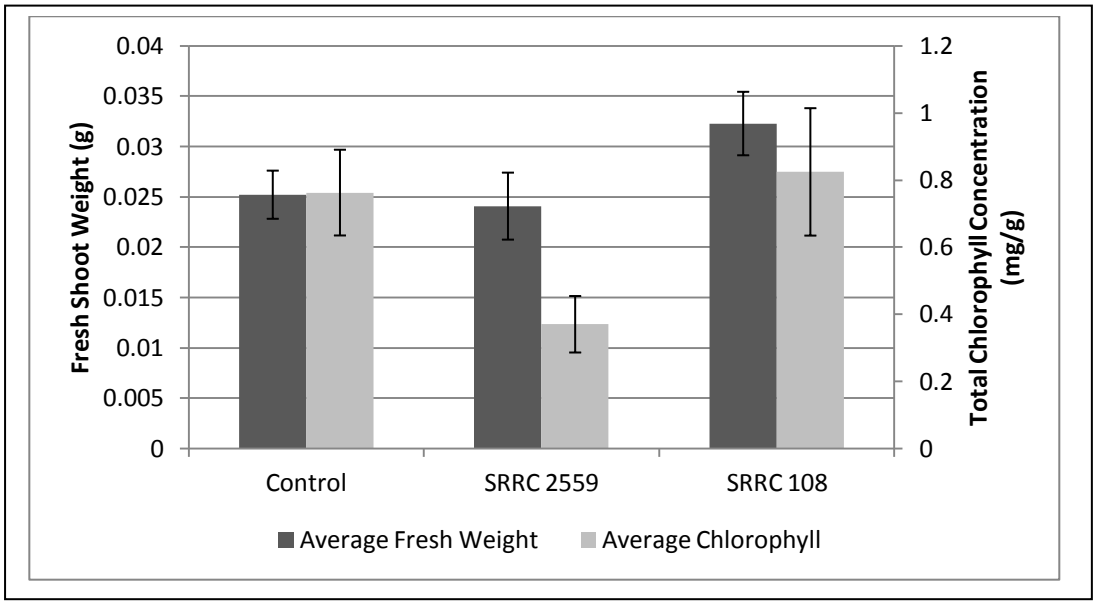

Figure 5. Average fresh weight and total chlorophyll concentration of *Arabidopsis thaliana* exposed to *Aspergillus* SRRC 2559 and SRRC 108 grown on YES media for 14 days. * denotes significance.

Effects of individual VOCs on seed germination and plant growth

Seed germination stages were classified into three groups: no germination, germination without seedling formation (Fig. 6C), and seedling formation (Fig. 6AB). The percentage of seeds that germinated and developed into seedlings in the presence of individual VOC is summarized in Figure 6. In the control, an average of 82% of seeds had undergone successful germination and progressed into the seedling stage. Of these, 7% exhibited various stages of seedling development (i.e. emergence of hook and cotyledons). The three terpenes tested, (limonene, β-farnesene, and β-caryophyllene) showed a seedling formation rate from 76- 79 $\pm$ 5%, with a significant increase in the 'germinated' rate and significant decrease in 'no germination' percentage compared to control seeds. Exposure to the alcohols 2-methyl-1-butanol and 3-methyl-1-butanol produced inhibitory effects and decreased seedling formation by 29% and 23%

respectively, a significant amount. Seeds exposed to these compounds had a higher germination rate than controls but many seeds only exhibited radical protrusion with no indication of hypocotyl elongation. The most inhibitory compound tested was 1-octen-3-ol. The percentage of non-germinated seeds was approximately 50%. Those seeds that did germinate (remaining 50%) all had radicles less than 1 mm in length (ANOVA, P = 0.01). At the end of the exposure period, seeds were removed from the exposure conditions and placed into clean, sterile plant media for an additional 72 hours without VOC exposure. Subsequent recovery and seedling formation were approximately 90% for all treatments (data not shown).

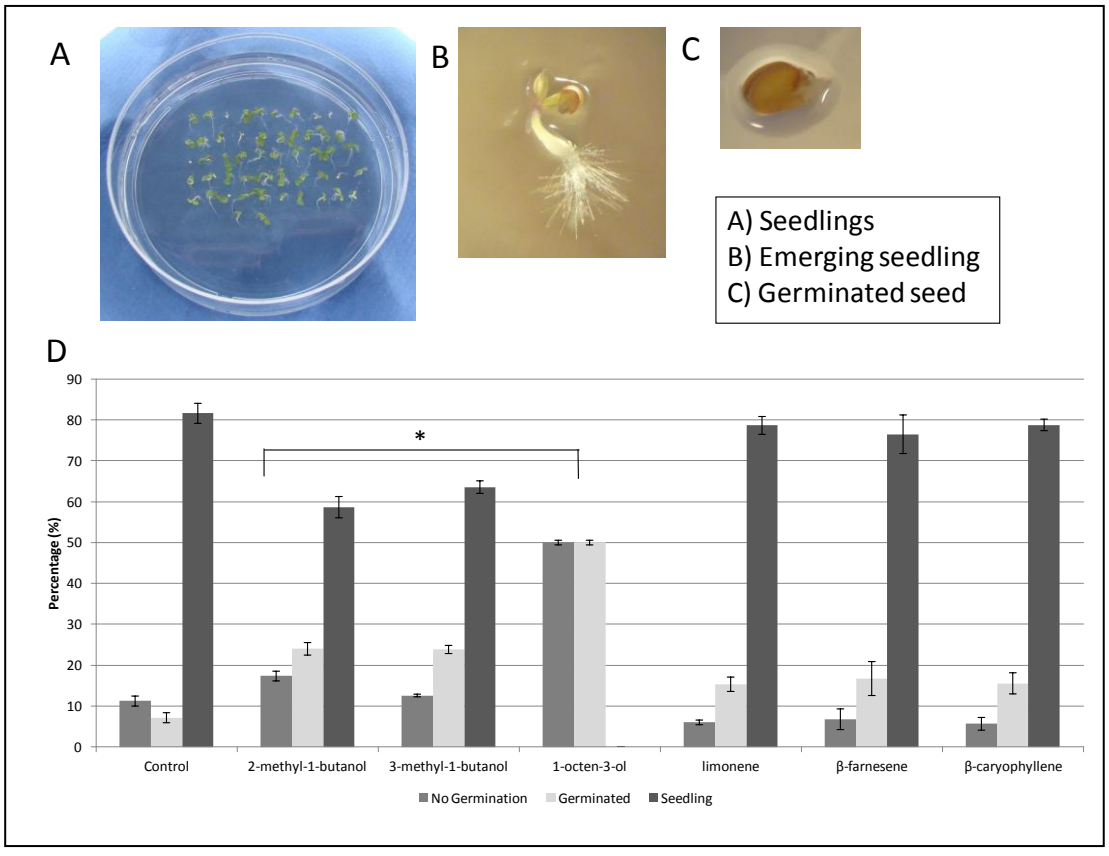

Figure 6. *Arabidopsis thaliana* seeds exposed to chemical standards, individual VOCs at 0.5 µl/l for 72 hours. Average percentage of seed germination and seedling development. * denotes significance.

Fourteen-day-old vegetative *Arabidopsis* plants were exposed to 0.5 µl/l of standard individual fungal VOC for 72 hours. The average fresh shoot weight and total chlorophyll concentration of controls and plants exposed to VOCs are summarized in Figure 7. Control plants were healthy with fully expanded green leaves with an average fresh shoot weight of 56.56 $\pm$ 3.72 mg. Plants exposed to each of the VOCs except 2-methyl-1-butanol exhibited either a significant increase or decrease in plant growth. Plants exposed to 1-octen-3-ol and β-farnesene exhibited inhibitory effects with smaller plant size and reduced total chlorophyll concentrations. Plants exposed to 1-octen-3-ol also had necrotic lesions on a few areas of the leaf, as well as yellowing of cotyledons and leaves. Plants exposed to 3-methyl-1-butanol, limonene, and β-caryophyllene showed significant increases in both plant biomass and total chlorophyll concentrations. The increase in fresh shoot weight ranged from 10 to 20% while total chlorophyll concentration increased 14 to 29% (ANOVA, $P = 0.01$). Root weights were similar in each testing condition (data not shown). Little or no purple pigment was observed in the underside of leaves when individual VOCs were tested.

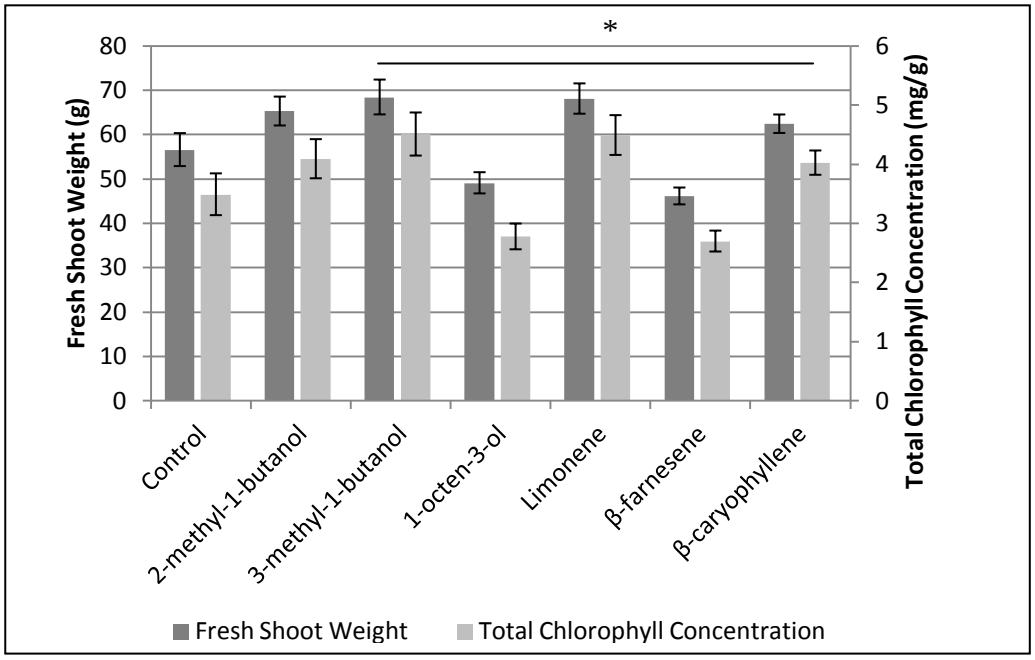

Figure 7. Average shoot fresh weight and total chlorophyll concentrations of *A. thaliana* exposed to chemical standards of VOCs at 0.5 µl/l for 72 hours. * denotes significance.

6.4 Discussion

The impact of nutrients, environmental conditions, and age of the colony on fungal growth and VOC profiles is well known (McNeal and Herbert, 2009; Insam and Seewald, 2010; Polizzi et al., 2012a; Lee et al., 2015). Furthermore, the collection and detection techniques used for obtaining VOC profiles also impact the results obtained (Hung et al., 2015). In our study of *A. versicolor* strains SRRC 108 and SRRC 2559 grown on YES medium, wallboard, and ceiling tile, both strains grew best and produced the largest number of detectable volatile compounds on YES. Both strains produced 2-methyl-1-butanol, 3-methyl-1-butanol, and 1-octen-3-ol on all three substrates, supporting the findings of others who have studied the VOCs of this species. In buildings with moisture and microbial problems, the reported levels of these individual compound

ranged from 0.1 - 10 µg/m^3. In some cases, 1-octen-3-ol was found at high concentration of up to 900 µg/m^3 and 3-methyl-1-butanol at 270 µg/m^3 (Korpi et al., 2009).

Using a passive sampling technique coupled with GC-MS, Matysik et al. (2009) found that *A. versicolor* grown on dichloran glycerol agar emitted 1,3-dimethoxybenzen, a compound not found from *A. fumigatus, A. niger* or several *Penicillium* species analyzed in their study. We did detected dimethoxybenzene in fungi growing on different substrates and for a shorter length of time.

Aspergillus versicolor produces sterigmatocystin, a hepatotoxic and nephrotoxic mycotoxin that is an intermediate of the aflatoxin biosynthetic pathway (Smith and Hacking, 1983; Rank et al., 2011). *A. versicolor* contamination is a problem in agriculture because this mold is commonly isolated from grains and green coffee beans (Vesonder and Horn, 1985; Pitt and Hocking, 2009). *A. versicolor* also is found in the built environment where its toxigenic potential has been hypothesized to contribute to health problems associated with damp indoor environments (Englehart, 2002). Aside from unpleasant odors, several volatile compounds have been associated with skin and airway irritations often associated with so-called "sick building syndrome" (Ezeonu et al., 1994). Fungal VOCs have been used as biomarkers in agriculture, medicine, and indoor air analyses. For example, they have been employed to detect the aflatoxin producing species *A. flavus* and *A. parasiticus* (Gao et al., 2002; Sun et al., 2014). Interestingly, some *A. flavus* VOCs affect fungal spore germination (Chippendale et al., 2014).

In our studies, comparison of the volatile profiles of the two *A. versicolor* strains showed an increased production of terpenes, sesquiterpenes, and diterpenes in SRRC 2559 relative to SRRC 108. The production of terpenes and terpenoid metabolites

originates in the synthesis of mevalonic acid (Heddergott et al., 2014). Several other studies have detected the production of sesquiterpenes by *Aspergillus* species (Korpi et al., 2009; Polizzi et al., 2012a; 2012b).

Since *A. versicolor* is a commonly isolated from soil and plant litter, we tested the impact of the VOCs made by the two strains on plant growth and seed germination. Using standardized protocols developed in our laboratory for exposing *A. thaliana* to fungal VOCs (Hung et al., 2013; Hung et al., 2014b; Lee et al., 2015), seeds and vegetative plants were exposed to volatile emissions from the two *A. versicolor* strains grown on YES. Seeds and plants exhibited differential responses between the two strains. While plants exposed to SRRC 108 were larger, the total chlorophyll content remained similar to the control. Plants exposed SRRC 2559 were similar in average fresh shoot weight relative to control, however, the plants had a significant reduction in chlorophyll content, indicating a stress response to the presence of VOCs.

Plants exposed to VOCs from both strains exhibited a reddish-purple coloration on the underside of the plant leaves, probably anthocyanin pigments. Anthocyanin production is a known plant response to unfavorable environmental conditions, hypothesized to allow plants to survive under occasional periods of harmful irradiation through modulation of light absorption (Das et al., 2011; Misyura et al., 2012). The VOC trans-2-hexenal has been shown to induce increased anthocyanin concentration in *Arabidopsis* (Bate and Rothstein, 1998). In summary, the decrease in total chlorophyll content and presence of pigment in the shoot indicates a stress response to the VOCs emitted by both strains of *A. versicolor*; however, the stress response was stronger with Strain 2559.

In an attempt to determine which compounds in the VOC mixture emitted by the two *A. versicolor* strains were responsible for our observed effects, we also evaluated plant response to six chemical standards at seed and vegetative growth stages. We tested three alcohols (2-methyl-1-propanol, 3-methyl-1-butanol, and 1-octen-3-ol) and two terpenes (limonene and β-farnesene) identified through our gas analysis. We also tested β-caryophyllene as a representative sesquiterpene for comparison. In our study, the alcohols were more inhibitory to seed germination and seedling formation than terpenes. Of the alcohols tested, 1-octen-3-ol was the most inhibitory. It caused seeds to arrest at germination, preventing further growth and development. It also was phytotoxic to vegetative plants causing localized death in plant tissue and overall reduction in chlorophyll. The antagonistic activities of 1-octen-3-ol have been reported previously (Splivallo et al., 2007; Junker and Tholl, 2013; Hung et al., 2014a; 2014b). It is important to note that the inhibitory effects observed on seed germination were not lethal; once the seeds were removed from the volatile treatment conditions they went on to complete germination and form seedlings. In contrast, at relatively low concentrations, exposing seeds to terpenes improved seed germination and seedling rate.

In vegetative plant exposure studies, differences were observed in plant size, leaf size and numbers, root growth, and chlorophyll concentration. Exposure to volatile phase 3-methyl-1-butanol, limonene, and β-caryophyllene improved plant growth, leading to larger, more robust plants with increased chlorophyll concentration. Exposure to volatile phase β-farnesene and 1-octen-3-ol reduced plant sizes and total chlorophyll concentration; plants exposed to 1-octen-3-ol exhibited additional symptoms of phytotoxicity such as curling of the leaf and presence of necrotic lesions. Localized cell

death in the plant tissue is indicative of oxidative burst caused by the overabundance of reactive oxygen species (ROS) (Heller and Tudzynski, 2011). Little or no purple pigment was observed in plants exposed to these individual compounds.

Most of the prior work on the effect of biogenic volatile phase compounds on plant growth has been conducted using bacteria from the rhizosphere (Ryu et al., 2003; Minerdi et al., 2009; Blom et al., 2011). Several rhizobacteria produce specific VOCs that have a positive impact on plant growth through direct stimulation of plant growth and/or reductions in the incidence of plant disease (Ryu et al., 2003; Vespermann et al., 2007; Zhang et al., 2008; Minerdi et al., 2011). By testing individual bacterial VOCs, Ryu et al. (2003) demonstrated that 2,3-butanediol and acetoin were both active in promoting plant growth. Farag et al. (2006) showed that several branched chain alcohols may also be involved in the growth stimulation effect.

Like bacteria, rhizosphere fungi may use volatile signaling to affect plant growth. Work in our lab has shown that VOCs from *Trichoderma*, a known biocontrol species, can improve growth in *A. thaliana* (Hung et al., 2013) and that low concentration of 2-methyl-1-butanol can yield a small but significant increase in fresh weight (Hung et al., 2014b). To our knowledge, this report is the first to show that VOCs from *A. versicolor* can have an impact on plant growth and seed germination.

In summary we have compared the VOC profiles of two strains of *A. versicolor*, assessed plant responses to the presence of their VOCs, and tested some individual VOCs from the mixture emitted by the two molds. Plants and their growth stages were differentially affected by the VOC mixtures emitted by the two strains with the VOCs from SRRC 2559 causing a strongly negative impact on *A. thaliana*. Anecdotally, it is of

interest that this strain came to our attention because it caused an irritant reaction in a student taking an *Aspergillus* identification workshop. Compounds such as 1-octen-3-ol may contribute to some of the decrease in plant growth that we observed. However, this single compound does not account for drastic reduction of chlorophyll in plants exposed to SRRC 2559. The difference in terpenes being produced by SRRC 2559 is evident and the biological activities of terpenes should be examined further.

REFERENCES

Bate, N.J., Rothstein, S.J. (1998). C6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. The Plant Journal. 16: 561-569.

Bennett, J.W., Hung, R., Lee, S., Padhi, S. (2012). Fungal and bacterial volatile organic compounds: an overview and their role as ecological signaling agents. In: Hock, B. (Ed.) The Mycota IX Fungal Interactions. Springer-Verlag. Pgs. 373-393.

Bitas, V., Kim, H.-S., Bennett, J., Kang, S. (2013). Sniffing on microbes: diverse roles of microbial volatile organic compounds in plant health. Molecular Plant-Microbe Interactions Journal. 4: 835-843.

Blom, D., Fabbri, C., Connor, E.C., Schiestl, F.P., Klauser, D.R., Boller, T., Eberl, L., Weisskopf, L. (2011). Production of plant growth modulating volatiles is widespread among rhizosphere bacteria and strongly depends on culture conditions. Environmental Microbiology. 13: 3047-3058.

Borjesson, T., Stollman, U., Adamek, P., Kaspersson, A. (1989). Analysis of volatile compounds for detection of molds in stored cereals. Cereal Chemistry Journal. 66: 300-304.

Boyes, D.C., Zayed, A.M., Ascenzi, R., McCaskill, A.J., Hoffman, N.E., Davis, K.R., Gorlach, J. (2001). Growth stage-based phenotypic analysis of *Arabidopsis*. The Plant Cell. 13: 1499-1510.

Chambers, S.T., Syhre, M., Murdoch, D.R., McCartin, F., Epton, M.J. (2009). Detection of 2-pentylfuran in the breath of patients with *Aspergillus fumigates*. Medical Mycology: Oxford Journals. 47: 468-476.

Chippendale, T.W.E., Gilchrist, F.J., Spanel, P., Alcock, A., Lenney, W., Smith, D., (2014). Quantification by SIFT-MS of volatile compounds emitted by *Aspergillus fumigatus* cultures and in co-culture with *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Streptococcus pneumoniae*. Analytical Methods. 6: 8154-8164.

Das, P.K., Geul, B., Choi, S.B., Yoo, S.D., Park, Y.I. (2011). Photosynthesis-dependent anthocyanin pigmentation in *Arabidopsis*. Plant Signaling & Behavior. 6: 23-25.

Ezeonu, I.M., Price, D.L., Simmons, R.B., Crow, S.A., Ahearn, D.G. (1994). Fungal production of volatiles during growth on fiberglass. Applied and Environmental Microbiology. 60: 4172-4173.

Engelhart, A., Loock, A., Skutlarek, D., Sagunski, H., Lommel, A., Farber, H., Exner, H. (2002). Occurrence of toxigenic *Aspergillus versicolor* isolates and sterigmatocystin in carpet dust from damp indoor environments. Applied and Environmental Microbiology. 68: 3886-3890.

Farag, M.A., Ryu, C.-M., Sumner, L.W., Pare, P.W. (2006). GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry. 67: 2262-2268.

Fischer, G., Schwalbe, R., Moller, M., Ostrowski, R., Dott, W. (1999). Species-specific production of microbial volatile organic compounds (MVOC) by airborne fungi from a compost facility. Chemosphere. 39: 795-810.

Fomicheva, G.M., Vasilenko, O.V., Marfenina, O.E. (2006). Comparative, morphological ecological and molecular studies of *Aspergillus versicolor* (Vuill.) Tiraboschi strains isolated from different ecotopes. Mikrobiologiia. 75: 186-191.

Gao, P., Korley, F., Martin, J., Chen, B. (2002). Determination of unique microbial volatile organic compounds produced by five *Aspergillus* species found in problem buildings. American Industrial Hygiene Association Journal. 63: 135-140.

Goldman, G.H., Osmani, S.A. (Ed.) (2008). The Aspergilli: Genomics, Medical Aspects, Biotechnology, and Research Methods. CRC Press Taylor & Francis Group.

Heddergott, C., Calvo, A.M., Latge, J.P. (2014). The volatome of *Aspergillus fumigatus*. Eukarotic Cell. 13: 1014-1025.

Heller, J., Tudzynski, P. (2011). Reactive oxygen species in phytopathogenic fungi: signaling, development, and disease. Annual Review of Phytopathology. 49: 369-390.

Hodgson, M.J., Morey, P., Leung, Y., Morrow, L., Miller, D., Jarvis, B.B., Robbins, Halsey J.F., Storey, E. (1998). Building-associated pulmonary disease from exposure to *Stachybotrys chartarum* and *Aspergillus versicolor*. Journal of Occupational and Environmental Medicine. 40: 241-249.

Hung, R., Lee, S., Bennett, J.W. (2013). *Arabidopsis thaliana* as a model system for testing the effects of *Trichoderma* volatile organic compounds. Fungal Ecology. 6: 19-26.

Hung, R., Lee, S., Bennett, J.W. (2014). The effects of low concentrations of the enantiomers of mushroom alcohol (1-octen-3-ol) on *Arabidopsis thaliana*. Mycology: An International Journal of Fungal Biology. 5: 73-80.

Hung, R., Lee, S., Bennett, J.W. (2015). Fungal volatile organic compounds and their role in ecosystems. Applied Microbiology and Biotechnology. 99: 3395-3405.

Hung, R., Lee, S., Rodriguez-Saona, C., Bennett, J.W. (2014). Common gas phase molecules from fungi affect seed germination and plant health in *Arabidopsis thaliana*. AMB Express. 4: 53. doi:10.1186/s13568-014-0053-8.

Insam, H., Seewald, S.A. (2010). Volatile organic compounds (VOCs) in soils. Biology and Fertility of Soils. 46: 199-213.

International Agency for Research on Cancer. (1987). Some naturally occurring substances. Monographs. 10: 245-251.

Junker, R.R., Tholl, D. (2013). Volatile organic compound mediated interactions at the plant-microbe interface. Journal of Chemical Ecology. 39: 810-825.

Jurjevic, Z., Rains, G.C., Wilson, D.M., Lewis, W.J. (2009). Volatile metabolites associated with one aflatoxigenic and one nontoxigenic *Aspergillus flavus* strain grown on two different substrates. Phytopathologia Mediterranea. 47: 266-271.

Korpi, A., Jarnberg, J., Pasanen, A.L. (2009). Microbial volatile organic compounds. Critical Reviews in Toxicology. 39: 139-193.

Latge, J.P. (1999). *Aspergillus fumigatus* and aspergillosis. Clinical Microbiology Reviews. 12: 310-350.

Lee, S., Hung, R., Schink, A., Mauro, J., Bennett, J.W. (2014). Phytotoxicity of volatile organic compounds. Plant Growth Regulation. 74: 177-186.

Lee, S., Hung, R., Yap, M., Bennett, J.W. (2015). Age matters: the effects of volatile organic compounds emitted by *Trichoderma atroviride* on plant growth. Archives of Microbiology. 197: 723-727.

Matysik, S., Herbarth, O., Mueller, A. (2009). Determination of microbial volatile organic compounds (MVOCs) by passive sampling onto charcoal sorbents. Chemosphere. 76: 114-119.

McNeal, K.S., Herbert, B.E. (2009). Volatile organic metabolites as indicators of soil microbial activity and community composition shifts. Soil Science Society of America Journal. 73: 579-588.

Machida, M., Gomi, K. (Ed.) (2010). *Aspergillus* Molecular Biology and Genomics. Caister Academic Press.

Minerdi, D., Bossi, S., Gullino, M.L., Garibaldi, A. (2009). Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. Environmental Microbiology. 11: 844-854.

Misyura, M., Colsanti, J., Rothstein, S.J. (2012). Physiological and genetic analysis of *Arabidopsis thaliana* anthocyanin biosynthesis mutants under chronic adverse environmental conditions. Journal of Experimental Botany: Oxford Journal. 64: 229-240.

Pasanen, P., Korpi, A., Kalliokosi, P., Pasanen, A.L. (1997). Growth and volatile metabolite production of *Aspergillus versicolor* in house dust. Environmental International. 23: 425-432.

Perrone, G., Susca, A., Cozzi, G., Ehrlich, K., Varga, J., Frisvad, J.C., Meijer, M., Noonim, P., Mahakarnchanakul, W., Samson, R.A. (2007). Biodiversity of *Aspergillus* species in some important agricultural products. Studies in Mycology. 59: 56-66.

Pitt, J.I., Hocking, A.D. (2009). The ecology of fungal food spoilage. In: Pitt, J.I. (Ed.) Fungi and Food Spoilage, 3rd edition. Springer. Pgs. 3-9.

Polizzi, V., Adams, A., De Saeger, S., Peteghem, C.S., Moretti, A., De Kimpe, N. (2012). Influence of various growth parameters on fungal growth and volatile metabolite production by indoor molds. Science of the Total Environment. 414: 277-286.

Polizzi, V., Adamns, A., Malysheva, S.V., De Saeger, S., Van Peteghem, C., Moretti, A., Picco, A.M., De Kimpe, N. (2012). Identification of volatile markers for indoor fungal growth and chemotaxonomic classification of *Aspergillus* species. Fungal Biology. 116: 941-953.

Rank, C., Nielsen, K.F., Larsen, T.O., Varga, J., Samson, R.A., Frisvad, J.C. (2011). Distribution of sterigmatocystin in filamentous fungi. Fungal Biology. 115: 406-420.

Roze, L.V., Beaudry, R.M., Arthur, A.E., Calvo, A.M., Linz, J.E. (2007). *Aspergillus* volatiles regulate aflatoxin synthesis and asexual sporulation in *Aspergillus parasiticus*. Applied Environmental Microbiology. 73: 7268-7276.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wie, H.-X., Pare, P.W., Kloepper, J.W. (2003). Bacterial volatiles promote growth of *Arabidopsis*. Proceedings of the National Academy of Sciences. 100: 4927-4932.

Smith, J.E., Hacking, A. (1983). Fungal toxicity. In: Smith, J.E., Berry, D.R., Kristiansen, B. (Ed.) The Filamentous Fungi Vol. IV. Edward Arnold Ltd. Pgs. 238-265.

Samson, R., Houbraken, B., Flannigan, M., Verhoeff, A., Adan, O., Hoekstra, E. (1994). Health implications of fungi in indoor environments. Air Quality Monographs Vol. 2. Elsevier. Pgs. 529-556.

Schleibinger, H., Laussmann, D., Brattig, C., Mangler, M., Eis, D., Ruden, H. (2005). Emission patterns and emission rates of MVOC and the possibility for predicting hidden mold damage? Indoor Air. 15: 98-104.

Splivallo, R., Novero, M., Bertea, C.M., Bossi, S., Bonfante, P. (2007). Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist. 175: 417-424.

Sun, D., Wood-Jones, A., Wang, W., Vanlangenberg, C., Jones, D., Gower, J., Simmons, P., Baird, R.E., Mlsna, T.E. (2014). Monitoring MVOC profiles over time from isolates of *Aspergillus flavus* using SPME GC-MS. Journal of Agricultural Chemistry and Environment. 3: 48-63.

Vespermann, A., Kai, M., Piechulla, B. (2007). Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Applied Environmental Microbiology. 73: 5639-5641.

Vesonder, R.F., Horn, B.W. (1985). Sterigmatocystin in dairy cattle feed contaminated with *Aspergillus versicolor*. Applied Environmental Microbiology. 49: 234-235.

Wessen, B., Strom, G., Palmgren, U., Schoeps, K., Nilsson, M. (2001). Analysis of microbial volatile organic compounds. In: Flannigan, B., Samson, R.A., Miller, J.D. (Ed.) Microorganisms in Home and Indoor Work Environments. Taylor & Francis. Pgs. 267-274.

Zhang, H., Xie, X., Kim, M., Kornyeyev, D.A., Holaday, S., Pare, P.W. (2008). Soil bacteria augment *Arabidopsis* photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. The Plant Journal. 56: 264-273.

Chapter 7.

Conclusions

Although fungal VOCs have been studied for decades, the recognition that they have a role in plant microbial ecology is relatively new. In agriculture, *Trichoderma* species are widely used as biocontrol agents that serve as plant growth promoters and for protection against pathogens. However, to date research on *Trichoderma* VOC production has been limited. Previous studies have focused on a very few isolates and were conducted with different species, differing in age of the culture, growth media, and VOC analytical methods. The plethora of fungal strains, experimental conditions and assay techniques makes it difficult to compare across studies. I have utilized *Arabidopsis thaliana* as a model to screen a large number of representative species of *Trichoderma* commonly isolated from the environment. My data demonstrates that *Trichoderma* volatile-induced plant growth promotion differs among individual isolates, and that the degree of plant growth promotion or inhibition in *Arabidopsis* is dependent on the environmental and physiological conditions of the fungi and plants. In addition, *Trichoderma*-derived VOCs induced similar plant growth promotion in tomato, with a significant increase in plant biomass, larger plant size, and significant development of lateral roots. This suggests that plant growth promotion may occur through a similar mode of action in different types of plants.

GC-MS analysis of 19 *Trichoderma* species and strains identified over 147 compounds and several unknown terpenes. While several compounds (2-methyl-1-propanol, acetoin, 2-butanone, acetone, 3-methyl-1-butanol, and 2-methylbutan-1-ol) were consistently produced by all isolates examined, unique compounds (2-heptylfuran, β-phellandrene, 2-methyl-5-propan-2-ylcyclohexan-1-one, (3E)-1,3-octadiene, and 1-decene) were produced by some isolates. Twenty six chemical standards, representing

common and uncommon fungal VOCs, were evaluated for their effects on *A. thaliana* seeds and vegetative plants growth. Several individual compounds, 3-methyl-1-butanol, 1-decene, and 2-heptylfuran, were able to induce similar plant growth promoting effects at 0.5 µg and 10 ng concentrations. The alkene, 1-decene, promoted *Arabidopsis* growth at relatively high (0.5 µg) and low (10 ng) concentrations, induced moderate improvement to germination, and caused the greatest increase in plant fresh weight and total chlorophyll content. It was selected for plant gene expression studies. RNA sequencing data identified 6 candidate genes related to growth and inducible by plant hormone auxin. Interestingly, genes related to plant defense (11 genes) and stress (23 genes) responses were down regulated in this study. Down regulation of defense response related genes in plants during the plant-microbe interactions have been reported previously. It is possible that *Trichoderma*-derived VOCs have two roles: 1) they work as biostimulatory compound to enhance plant growth leading to increase production of nutrient for the fungi, 2) they assist in reducing the plant immunity temporarily in order for the fungi to successfully colonize the host plant.

Our current knowledge of the VOCs emitted by microorganisms will continue to grow as we obtain new volatile profiles, accurately assess the impact of environmental conditions on volatile production, and identify novel compounds. A critical step in the improved practical application of these VOCs is to develop a mechanistic understanding of the volatile-mediated *Trichoderma*-to-plant interactions. The candidate 1-decene-responsive genes identified in this study can be used to devise future experiments with the intent of shedding light into specific pathways that may be affected by VOC

exposure. The exploitation of *Trichoderma* volatiles as plant growth promoters and/or disease suppressors has the potential to become a powerful tool in agriculture.

APPENDIX

Arabidopsis thaliana as a Model System for Testing the Effect of *Trichoderma* VOCs

Hung, R., Lee, S., Bennett, J.W. (2013). Fungal Ecology. 6: 19-26.

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 and Tomlinson, 1999; Pichersky and 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 and Michelet, 2005; Kues and 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 (Windham et al., 1986; Contreras-Cornejo et al., 2009). Several groups have shown that the VOCs of different *Trichoderma* species can inhibit other fungi, in particular wood decay basidiomycetes and plant pathogens (Dennis and 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 ^{o}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 ^{o}C ± 2 ^{o}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.

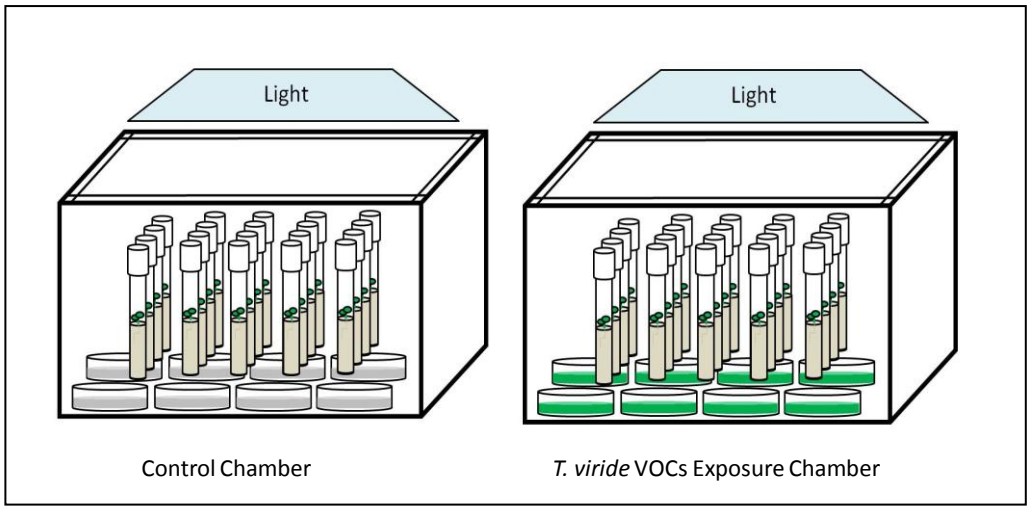

Figure 1. *Trichoderma viride* VOC exposure chamber setup.

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 is volume and W is 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_2O_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^{o}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^{o}C to 280^{o}C at 10^{o}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 (Fig. 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 (Fig. 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.

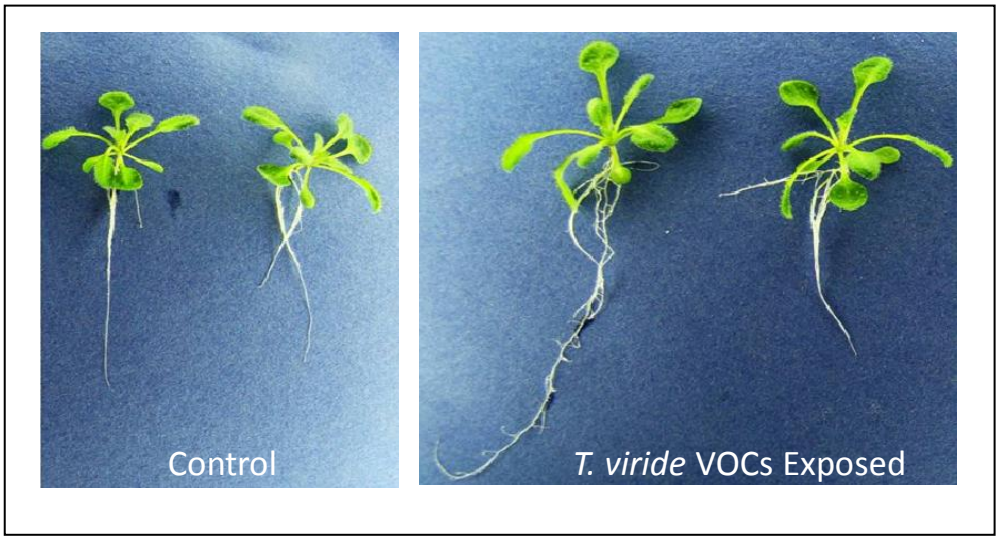

Figure 2. *Arabidopsis thaliana* exposed to *Trichoderma viride* VOCs for two weeks. The visible indicators of growth promotion in volatile exposed plants were: larger leaves and root mass and increased lateral root development.

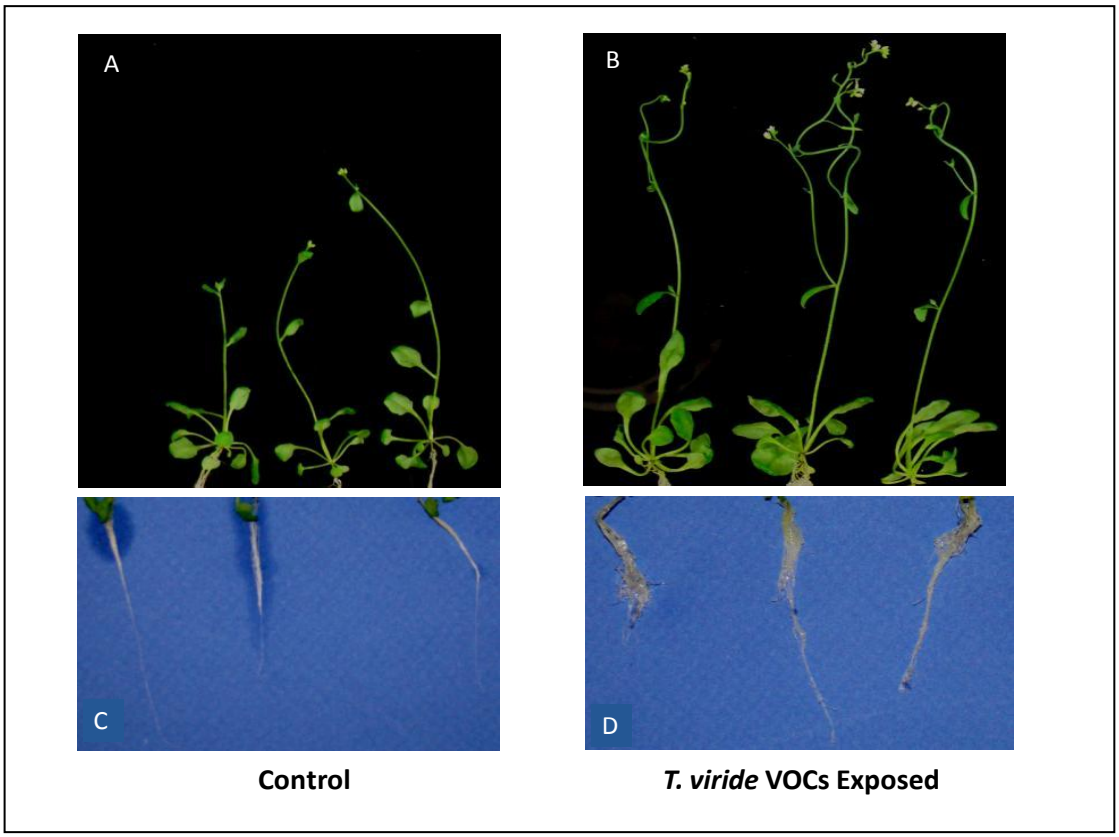

Figure 3. *Arabidopsis thaliana* exposed to *Trichoderma viride* VOCs for four weeks. (A) Control plants and (B) *A. thaliana* exposed to *T. viride* have visible indicators of growth promotion including larger leaves, taller flower stems, formation of several flowers, and earlier flowering. (C) Roots of control and (D) Roots of *A. thaliana* exposed to *T. viride* have a more robust root mass and increased lateral root development.

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.

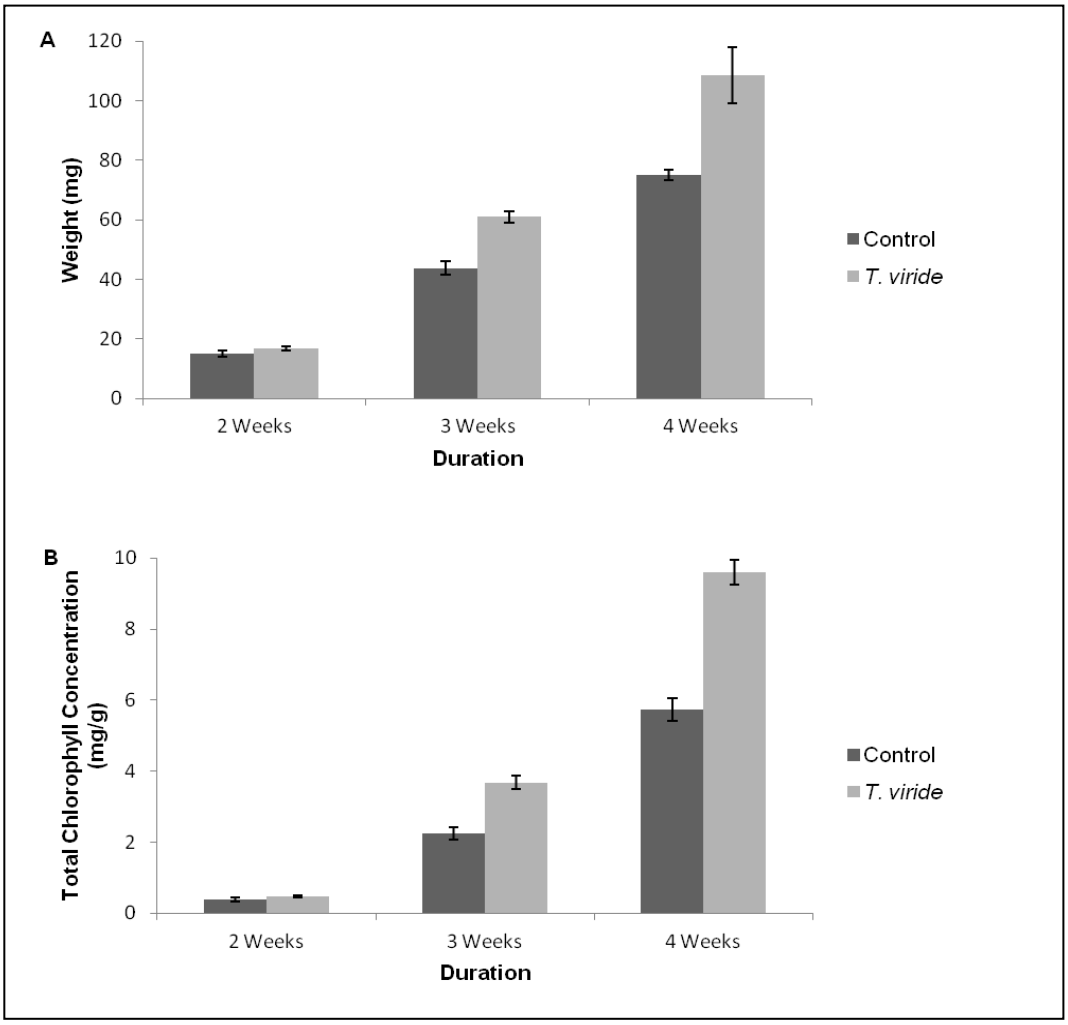

Figure 4. *Arabidopsis thaliana* exposed to *Trichoderma viride* VOCs for four weeks. (A) Weekly average fresh weight of above-ground plant mass. (B) Total chlorophyll concentration of above-ground plant mass of *A. thaliana*. The data collected represents the averages of 50 plants per condition repeated 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 fresh weight.

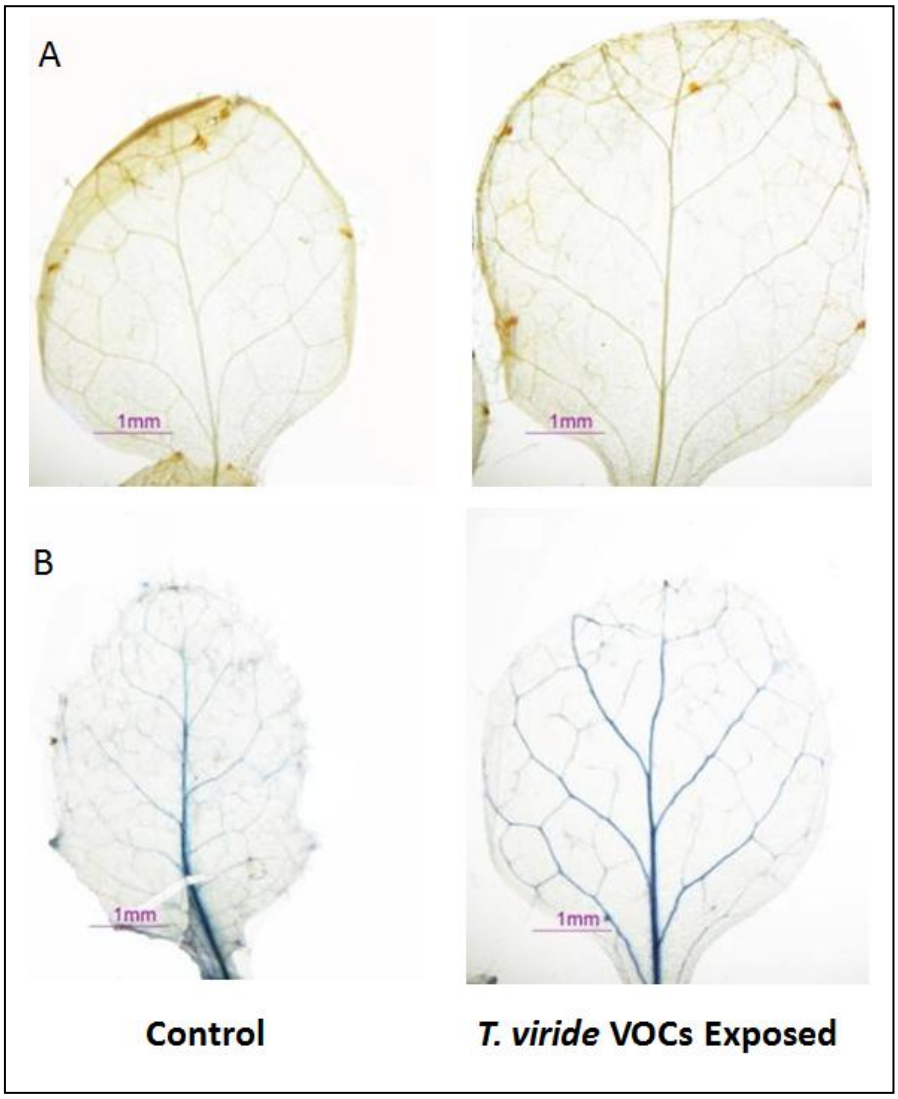

Figure 5. *Arabidopsis thaliana* exposed *Trichoderma viride* VOCs for two weeks. (A) DAB staining of *A. thaliana* leaves. Plants exposed to *T. viride* VOCs and control plants have similar amount of hydrogen peroxide. (B) Trypan blue staining of *A. thaliana* leaves. Both plant leaves look similar.

MS Scan #	Peak Assignment
156	Ethanol
189	Acetone
285	2-methylpropanal
338	Butanal
380	isobutyl alcohol
405	3-methylbutanal
416	2-methylbutanal
423	1-butanol
441	2-pentanone
455	Pentanal
489	Acetoin
493	2-methyl-1-butanol
497	isopentyl alcohol
525	d-8 toluene (internal standard)
549	octane
558	Hexanal
583	Octadiene
619	branched nonane isomer
644	2-heptanone
654	Heptanal
702	2-ethylhexanal
728	6-methyl-5-hepten-2-one
731	1-decene
734	2-pentylfuran
738	2-octanone
742	Octanal
773	Limonene
787	branched C12 paraffin
792	branched C12 paraffin
805	trans-2-octenal
808	octyl alcohol
818	n-undecane
823	Nonanal
890	2-heptylfuran
898	Decanal
903	d-8 naphthalene (internal standard)
936	cyclohexaneisothiocyanate
950	branched C15 paraffin
960	branched C15 paraffin
968	branched C15 paraffin
979	785 branched C15 paraffin
985	233 branched C15 paraffin
990	201 branched C15 paraffin
1065	332 geranyl acetone
1069	204 m.w. sesquiterpene
1101	beta-himachalene
1107	Farnescene
1119	aromadendrene
1125	204 m.w. sesquiterpene
1135	204 m.w, sesquiterpene
1202	222 m.w. sesquiterpene
1371	pimar-8,15-diene
1382	kaur-15-ene

Table1. Headspace volatiles collected from 7 day old *Trichoderma viride* 100 ml/min. Purge Rate, 4 hours, 1.0 µg Int. Std. by P&T-TD-GC-MS.

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 anti-fungal 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 *Muscodor* 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* (Sivan and Chet, 1989; Ahmed et al., 1999; Harman et al., 2004). *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 (Elad et al., 1982; Cherif and Benhamou, 1990; Sahai and Manocha, 1993; Inbar and Chet, 1995; Haran et al., 1996). 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, *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 (Zhang et al., 2008; Minerdi et al., 2011). 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.

REFERENCES

Ahmed, A.S., Perez-Sanchez, C., Egea, C., Candela, M.E. (1999). Evaluation of *Trichoderma harzianum* for controlling root rot caused by *Phytopthora capsici* in pepper plants. Plant Pathology. 48: 58-65.

Benítez, T., Rincón, A.M., Limón, M.C., Codón, A.C. (2004). Biocontrol mechanisms of *Trichoderma* strains. International Microbiology. 7: 249-260.

Bennett, J.W., Hung, R., Lee, S., Padhi, S. (2012). Fungal and bacterial volatile organic compounds: an overview and their role as ecological signaling agents. In: Hock, B. (Ed.) The Mycota IX Fungal Interactions. Springer-Verlag, Heidelberg. Pgs. 373-393.

Boddy, L., Frankland, J.C., Vest, P. (2008). Ecology of saprophytic basidiomycetes. Elsevier, Oxford.

Bruce, A., Verrall, S., Hackett, C.A., Wheatley, R.E. (2004). Identification of volatile organic compounds (VOCs) from bacteria and yeast causing growth inhibition of sapstain fungi. Holzforschung. 58: 193-198.

Carter, J.P., Whipps, J.M., Lynch, J.M. (1990). Comparison of biomass and viable propagule measurements in the antagonism of *Trichoderma harzianum* against *Pythium ultimum*. Soil Biology & Biochemistry. 22: 187-194.

Cherif, M., Benhamou, N. (1990). Cytochemical aspects of chitin breakdown during the parasitic action of a *Trichoderma* sp. on *Fusarium oxysporum* f. sp. r*adicis-lycopersici*. Phytopathology. 80: 1406-1414.

Chiron, N., Michelot, D. (2005). Odeurs des champignons: chimie et rôle dans les interactions biotiques - une revue = Mushrooms odors, chemistry and role in the biotic interactions: a review. Cryptogamie Mycoogiel. 26: 299-364.

Claeson, A.S., Levin, J.O., Blomquist, G., Sunesson, A.L. (2002). Volatile metabolites from microorganisms grown on humid building materials and synthetic media. Journal of Environmental Monitoring. 4: 667-672.

Contreras-Cornejo, H.A., Macias-Rodriguez, L.I., Cortes-Penagos, C., Lopez-Bucio, J. (2009). *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in *Arabidopsis*. Plant Physiology. 149: 1579-1592.

Dennis, C., Webster, J. (1971). Antagonistic properties of species-groups of *Trichoderma* 11. Production of volatile antibiotics. Transactions of the British Mycological Society. 57: 41-48.

Dighton, J., White, J.F., Oudemans, P. (2005). The fungal community: its organization and role in the ecosystem. Taylor and Francis Group.

Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E., Mukherjee, P.K., Zeilinger, S., Grigoriev, I.V., Kubicek, C.P. (2011). *Trichoderma*: the genomics of opportunistic success. Nat Reviews Microbiology. 9: 749-759.

Elad, Y., Chet, I., Henis, Y. (1982). Degradation of plant pathogenic fungi by *Trichoderma harzianum*. Canadian Journal of Microbiology. 28: 719-725.

Farag, M.A., Ryu, C.-M., Sumner, L.W., Pare, P.W. (2006). GC-MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants. Phytochemistry. 67: 2262-2268.

Fernando, W.G.D., Ramarathnam, R., Krishnamoorthy, A.S., Savchuk, S.C. (2005). Identification and use of potential bacterial organic antifungal volatiles in biocontrol. Soil Biology & Biochemistry. 37: 955-964.

Hansen, V.M., Winding, A., Madsen, A.M. (2010). Exposure to bioaerosols during the growth season of tomatoes in an organic greenhouse using Supresivit (*Trichoderma harzianum*) and Mycostop (*Streptomyces griseoviridis*). Applied Environmental Microbiology. 76: 5874-5881.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M. (2004). *Trichoderma* species-opportunistic, avirulent plant symbionts. Nature Reviews Microbiology. 2: 43-56.

Haran, S., Schickler, H., Chet, I. (1996). Molecular mechanisms of lytic enzymes involved in the biocontrol activity of *Trichoderma harzianum*. Microbiology. 142: 2321-2331.

Henis, Y., Lewis, J.A., Papavizas, G.C. (1984). Interactions between *Sclerotium rolfsii* and *Trichoderma* spp: Relationship between antagonism and disease control. Soil Biology & Biochemistry. 16: 391-395.

Herrmann, A. (2010). The Chemistry and Biology of Volatiles. John Wiley & Sons.

Humphris, S.N., Bruce, A., Buultjens, E., Wheatley, R.E. (2002). The effects of volatile microbial secondary metabolites on protein synthesis in *Serpula lacrymans*. FEMS Microbiology Letters. 210: 215-219.

Humphris, S.N., Wheatley, R.E., Bruce, A. (2001). The effects of specific volatile organic compounds produced by *Trichoderma* spp. on the growth of wood decay basidiomycetes. Holzforschung. 55: 233-237.

Hynes, J., Müller, C.T., Jones, T.H., Boddy, L. (2007). Changes in volatile production during the course of fungal mycelial interactions between *Hypholoma fasciculare* and *Resinicium bicolor*. Journal of Chemical Ecology. 33: 43-57.

Inbar, J., Chet, I. (1995). The role of recognition in the induction of specific chitinases during mycoparasitism by *Trichoderma harzianum*. Microbiology. 141: 2823-2829.

Jing, H., Sturre, M.J.G., Hille, J., Dijkwel, P.P. (2002). *Arabidopsis* onset of leaf death mutants identify a regulatory pathway controlling leaf senescence. The Plant Journal. 32: 51-63.

Kai, M., Crespo, E., Cristescu, S.M., Harren, F.J.M., Piechulla, B. (2010). *Serratia odorifera*: analysis of volatile emission and biological impact of volatile compounds on *Arabidopsis thaliana*. Applied Microbiology and Biotechnology. 88: 965-976.

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., Piechulla, B. (2009). Bacterial volatiles and their action potential. Applied Microbiology and Biotechnology. 81: 1001-1012.

Kishimoto, K., Matsui, K., Ozawa, R., Takabayashi, J. (2007). Volatile 1-octen-3-ol induces a defensive response in *Arabidopsis thaliana*. Journal of Plant Pathology. 73: 35-37.

Korolev, N., David, D.R., Elad, Y. (2008). The role of phytohormones in basal resistance and *Trichoderma*-induced systemic resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. BioControl. 53: 667-683.

Korpi, A., Jarnberg, J., Pasanen, A.L. (2009). Microbial volatile organic compounds. Critical Reviews in Toxicology. 39: 139-193.

Krupke, O.A., Castle, A.J., Rinker, D.L. (2003). The North American mushroom competitor, *Trichoderma aggressivum* f. *aggressivum*, produces antifungal compounds in mushroom compost that inhibit mycelial growth of the commercial mushroom *Agaricus bisporus*. Mycological Research. 107: 1467-1475.

Kues, U., Navarro-Gonzales, M. (2009). Communication of fungi on individual, species, kingdom, and above kingdom levels. In: Anke, T., Weber, D., (Ed.) The Mycota XV Physiology and Genetics. Springer-Verlag. Pgs. 79-106.

Leopold, A.C. (1964). Plant Growth and Development. McGraw-Hill Book Co.

Minerdi, D., Bossi, S., Gullino, M.L., Garibaldi, A. (2009). Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. Environmental Microbiology. 11: 844-854.

Minerdi, D., Bossi, S., Maffei, M.E., Gullino, M.L., Garibaldi, A. (2011). *Fusarium oxysporum* and its bacterial consortium promote lettuce growth and expansin A5 gene expression through microbial volatile organic compound (MVOC) emission. FEMS Microbiology Ecology. 76: 342-351.

Mølhave, L. (2009). Volatile organic compounds and the sick building syndrome. In: Lippmann, M. (Ed.) Environmental Toxicants: Humans Exposures and Their Health Effects, 3rd edition. Wiley-Interscience. Pgs. 241-254.

Ousley, M.A., Lynch, J.M., Whipps, J.M. (1994). The effects of addition of *Trichoderma* inocula on flowering and shoot growth of bedding plants. Scientia Horticulturae. 59: 147-155.

Palta, J.P. (1990). Leaf chlorophyll content. Remote Sensing Reviews. 5: 207-213.

Pare, P.W., Tumlinson, J.H. (1999). Plant volatiles as a defense against insect herbivores. Plant Physiology. 121: 325-331.

Pichersky, B., Gershenzon, J. (2002). The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Current Opinion in Plant Biology. 5: 237-243.

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Wie, H.-X., Pare, P.W., Kloepper, J.W. (2003). Bacterial volatiles promote growth of *Arabidopsis*. Proceedings of the National Academy of Sciences. 100: 4927-4932.

Sahai, A.S., Manocha, M.S. (1993). Chitinases of fungi and plants: their involvement in morphogenesis and host-parasite interaction. FEMS Microbiology Reviews. 11: 317-338.

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. Phytopathology. 95: 76-84.

Sivan, A., Chet, I. (1989). Degradation of fungal cell walls by lytic enzymes of *Trichoderma harzianum*. Microbiology. 135: 675-682.

Splivallo, R., Novero, M., Bertea, C.M., Bossi, S., Bonfante, P. (2007). Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist. 175: 417-424.

Splivallo, R., Fischer, U., Gobel, C., Feussner, I., Karlovsky, P. (2009). Truffles regulate plant root morphogenesis via the production of auxin and ethylene. Plant Physiology. 150: 2018-2029.

Splivallo, R., Ottonello, S., Mello, A., Karlovsky, P. (2011). Truffle volatiles: from chemical ecology to aroma biosynthesis. New Phytologist. 189: 688-699.

Steinebrunner, F., Schiestl, F.P., Leuchtmann, A. (2008). Ecological role of volatiles produced by *Epichloe*: differences in antifungal toxicity. FEMS Microbiology Ecology. 64: 307-316.

Strobel, G. (2006). *Muscodor albus* and its biological promise. Journal of Industrial Microbiology & Biotechnology. 33: 514-522.

Strobel, G.A., Dirkse, E., Sears, J., Markworth, C. (2001). Volatile antimicrobials from *Muscodor albus*, a novel endophytic fungus. Mirobiology. 147: 2943-2950.

Sunesson, A.-L., Vaes, W.H.J., Nilsson, C.-A., Blomquist, G.R., Andersson, B., Carlson, R. (1995). Identification of volatile metabolites from five fungal species cultivated on two media. Applied Environmental Microbiology. 61: 2911-2918.

Thordal-Christensen, H., Zhang, Z., Wei, Y., Collinge, D.B. (1997). Subcellular localizastion of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-mildew interaction. The Plant Journal. 11: 1187-1194.

Yedidia, I., Benhamou, N., Chet, I. (1999). Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. Applied Environmental Microbiology. 65: 1061-1070.

van Wees, S. (2010). Phenotypic analysis of *Arabidopsis* mutants: trypan blue stain for fungi, oomycetes and dead plant cells. Cold Spring Harbor Protocols.

Vespermann, A., Kai, M., Piechulla, B. (2007). Rhizobacterial volatiles affect the growth of fungi and *Arabidopsis thaliana*. Applied Environmental Microbiology. 73: 5639-5641.

Wheatley, R.E. (2002). The consequences of volatile organic compound mediated bacterial and fungal interactions. Antonie van Leeuwenhoek. 81: 357-364.

Wheatley, R., Hackett, C., Bruce, A., Kundzewicz, A. (1997). Effect of substrate composition on production of volatile organic compounds from *Trichoderma* spp. inhibitory to wood decay fungi. International Biodeterioration & Biodegradation. 39: 199-205.

Wilkins, K., Larsen, K. (1995). Variation of volatile organic compound patterns of mold species from damp buildings. Chemosphere. 31: 3225-3236.

Windham, M.T., Elad, Y., Baker, R. (1986). A mechanism for increased plant growth induced by *Trichoderma* spp. Phytopathology. 76: 518-521.

Zhang, H., Xie, X., Kim, M., Kornyeyev, D.A., Holaday, S., Pare, P. (2008). Soil bacteria augment *Arabidopsis* photosynthesis by decreasing glucose sensing and abscisic acid levels in planta. The Plant Journal. 56: 264-273.

Zogorski, J.S., Carter, J.M., Ivahnenko, T., Lapham, W.W., Moran, M.J., Rowe, B.L., Squillace, P.J., Toccalino, P.L. (2006). The quality of our nation's waters--volatile organic compounds in the nation's ground water and drinking-water supply wells. US Geological Survey Circular. 1292:101.