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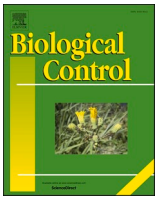

# Multifaceted effects of volatile organic compounds released by *Fusarium oxysporum* on *Trichoderma* biocontrol agents

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## HIGHLIGHTS

- Five volatile organic compounds produced by *Fusarium oxysporum* inhibit *Trichoderma* growth.
- *Trichoderma* responds to VOCs with altered secretion of antifungal molecules.
- Genes associated with biocontrol and other processes are affected by VOCs.

## ARTICLE INFO

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## ABSTRACT

We investigated how volatile organic compounds (VOCs) produced by *Fusarium oxysporum*, a soilborne fungal species complex that infects diverse plants, affect the growth of four *Trichoderma* spp. and their secretion of antifungal molecules and gene expression to evaluate the involvement of VOC-mediated recognition of pathogens in biocontrol. Five VOCs commonly produced by all *F. oxysporum* strains analyzed, including 4-ethylanisole, 1-hexanol, 3-methyl-1-butanol, isopentyl acetate, and 2-phenylethanol, were tested. All compounds inhibited *Trichoderma* growth in a concentration-dependent manner, with 4-ethylanisole being the most effective. Although the degree of growth inhibition by each compound was similar among the four species, their secretion of antifungal molecules varied widely, with the degree of induction in *T. virens* and *T. viride* being higher than that in *T. harzianum* and *T. asperellum* in most treatments. Transcriptome analyses via RNA-seq after exposure to 4-ethylasniole, 3-methyl-1-butanol, isopentyl acetate, and VOCs released by three *F. oxysporum* strains were performed. Analysis of gene ontology (GO) terms enriched among the differentially expressed genes (DEGs) revealed that diverse processes, such as synthesizing/metabolizing various types of organic compounds, ion/carbohydrate transport, proteolysis, response to stimulus, signal transduction, chromosome organization, RNA processing, and DNA metabolism, were significantly affected. Some genes likely involved in biocontrol, such as those that perform fungal cell wall degradation, protein hydrolysis, and secondary metabolite biosynthesis, were also differentially regulated. However, the DEGs in the four species did not overlap significantly, suggesting that individual species respond distinctly to each VOC treatment. Collectively, our data suggest that *Trichoderma* BCAs recognize other fungi through multiple specific volatile cues and prepare for subsequent encounters. We discuss future studies needed to test this hypothesis and to uncover the mechanism underlying the VOC-mediated recognition of pathogens and the resulting modulation of diverse cellular responses.

## 1. Introduction

The global need for affordable and nutritious foods continuously increases. Plant diseases challenge efforts to meet this need. Globally, the estimated yearly crop loss caused by pathogens is 13 % (Pimentel, 2009). Chemical pesticides have reduced this loss; however, pesticide residues threaten human and animal health and vital ecosystem services (Pimentel, 2009; Zhang, 2018; Zhang et al., 2011). Moreover, the extensive use of chemical pesticides selects for pesticide-resistant pathogen populations (Hahn, 2014). Biological control, which harnesses specific living organisms to suppress diseases, is an environment-friendly alternative to chemical pesticides. Members of the fungal genus

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*Trichoderma* occupy diverse ecological niches and suppress other organisms by using enzymatic and chemical weapons (Schmoll and Schuster, 2010; Zeilinger et al., 2016). Diverse *Trichoderma* spp. and their metabolites have been screened for biological control of pathogenic fungi, oomycetes, and nematodes (Druzhinina et al., 2011; Fan et al., 2020; Harman et al., 2004; Hyder et al., 2017). *Trichoderma* suppresses pathogens by employing parasitism and antibiosis, competing for space and nutrients, and inducing systemic resistance in plants (Harman et al., 2004; Howell, 2003; Sawant, 2014; Shoresh et al., 2010; Woo et al., 2014). Several *Trichoderma* spp. have also been used as biostimulants and biofertilizers because of their ability to solubilize various nutrients into forms that plants can efficiently acquire, increase nitrogen use efficiency, and modulate plant hormone signaling pathways to enhance seed germination and development (Contreras-Cornejo et al., 2009; Fiorentino et al., 2018; López-Bucio et al., 2015; Pascale et al., 2017; Singh et al., 2018).

Advances in genomics and molecular genetics have facilitated the identification and characterization of proteins and metabolites likely involved in biocontrol, including cell wall-degrading enzymes, proteases, and secondary (=specialized) metabolites (Marik et al., 2018; Samolski et al., 2009; Schmoll et al., 2016; Seidl et al., 2009; Suárez et al., 2007; Sun et al., 2016; Wu et al., 2017). In our previous study (Li et al., 2018), volatile organic compounds (VOCs) produced by *Fusarium oxysporum*, a soilborne fungal species complex causing vascular wilt or crown and root rot in more than 100 plant species (Gordon and Martyn, 1997), induced antifungal molecule secretion by some *Trichoderma* biological control agents (BCAs): *T. virens* and *T. viride* significantly increased antifungal molecule secretion upon exposure to VOCs produced by 13 *F. oxysporum* strains representing seven *formae speciales*; *T. harzianum* responded to VOCs produced by only some *F. oxysporum* strains; *T. asperellum* did not respond to any. We also found that VOCs released by phylogenetically diverse bacteria isolated from tomato roots affect the secretion of antimicrobial molecules by *Trichoderma* (Li et al., 2019). These findings support the hypothesis that *Trichoderma* modulates its response to neighboring microbes by recognizing specific microbial VOCs as signals or elicitors. VOC-mediated induction of antifungal molecule secretion was also observed in *F. oxysporum* when exposed to VOCs produced by *Trichoderma* (Li et al., 2018), suggesting that VOC-mediated recognition and response to other fungi are not limited to *Trichoderma*.

VOCs include various classes of compounds with high vapor pressure (0.01 kPa or higher at 20 °C), small molecular weight (<300 Dalton), and lipophilic/hydrophobic moieties. Volatility facilitates their movement through the atmosphere and porous soils, enabling some VOCs to function as intra/inter-species signals or chemical weapons (Bitas et al., 2013; Das et al., 2013; Junker and Tholl, 2013; Kanchiswamy et al., 2015; Kang et al., 2021; Li et al., 2016). Bacteria and fungi produce a wealth of VOCs, some of which have been shown to be involved in chemical warfare, quorum sensing, and regulating the growth and development of themselves and other organisms (Bennett and Inamdar, 2015; Bitas et al., 2013; Chen et al., 2016; Kang et al., 2021; Li et al., 2016; Quintana-Rodriguez et al., 2018; Rybakova et al., 2017). VOCs produced by *Straphylococcus sciuri* MarR44, an endophytic bacterial isolate associated with strawberries, inhibit mycelial growth and conidial germination of *Colletotrichum nymphaeae* (Alijani et al., 2019). In response to nitrogen starvation, *Saccharomyces cerevisiae* releases phenylethanol and tryptophol to trigger its morphological transition from unicellular yeasts to invasive filamentous hyphae (Chen and Fink, 2006). *Escherichia coli* genes involved in motility and antibiotic resistance are differentially regulated in response to VOCs produced by *Bacillus subtilis*, suggesting that *E. coli* uses volatile signals to sense neighboring microbes and regulate physiological responses (Kim et al., 2013).

Analysis of VOCs produced by multiple *F. oxysporum* strains via gas chromatography-mass spectrometry (GC–MS) showed that 4-ethylanisole (produced via the phenylalanine biosynthesis pathway), 1-hexanol (produced using acetyl-CoA), 3-methyl-1-butanol (produced via the leucine biosynthesis pathway), isopentyl acetate (produced via alcohol fermentation), and 2-phenylethanol (produced via the Ehrlich pathway) were commonly produced (Li et al., 2018), suggesting that these VOCs may act as elicitors recognized by *Trichoderma*. The aim of the present study was to determine whether *T. virens*, *T. harzianum*, *T. asperellum*, and *T. viride* modulate the secretion of antifungal molecules in response to these candidate VOC elicitors. We also analyzed how individual *Trichoderma* spp. reprogram transcriptomes in response to three selected compounds and whole VOCs produced by three *F. oxysporum* strains to investigate the mechanism of molecular responses to VOCs and whether VOC-mediated responses likely affect biocontrol efficacy.

## 2. Materials and methods

### 2.1. Fungal cultures

Four strains isolated from commercial biocontrol products (Li et al., 2018), *T. virens* G-41, *T. harzianum* T-22, *T. asperellum* ICC 012, and *T. viride* OSK-36, were used. *Fusarium oxysporum* f. sp. *lycopersici* strain NRRL54003 was obtained from the USDA-ARS Culture Collection at the National Center for Agricultural Utilization Research. All fungal strains were preserved as spore suspensions in 20 % glycerol at −80 °C and revitalized by inoculating on 0.75X (3/4 of the amount recommended by the manufacturer) potato dextrose agar (PDA; Difco, Houston, TX) at 25 °C without light.

### 2.2. Effect of individual VOCs on Trichoderma growth and antifungal molecule secretion

All compounds tested (Table A.1), except 4-ethylanisole (Alfa Aesar, Haverhill, MA), were purchased from Sigma-Aldrich (St. Louis, MO). A previously used method (Li et al., 2018; Li and Kang, 2022) was employed to determine the effect of each compound on *Trichoderma* growth and its secretion of antifungal molecules, as illustrated in Fig. 1. After inoculating a plug of freshly prepared *Trichoderma* culture on a sterile cellophane membrane (Paper Mart, Orange, CA) overlaid on 15 mL 0.75X PDA, the plate was immediately sandwiched with another plate containing each compound (Table A.1**)** at three concentrations (20, 100, and 500 μmol/L in the headspace of sandwiched plates). Because some compounds could dissolve the plastic Petri plate, we applied each compound to a sterile filter paper (Whatman, Clifton, NJ) disk (d = 6 mm) placed on a glass coverslip immediately before sandwiching the plates. The following equation was used to calculate the volume of each compound (V) required to achieve the target concentration:

$$V\,(uL) = \frac{0.15\,(L) \times Final\ concentration\ (\mu mol/L) \times MW\,(g/mol)}{Density\,(g/L)}$$

The headspace volume within the two sandwiched plates is 0.15 L. The control plates contained *Trichoderma* culture but no VOC.

The sandwiched plates were sealed with three layers of Parafilm and placed randomly in an incubator at 25 °C for 33 hr (*T. harzianum* and *T. asperellum*) or 40 hr (*T. virens* and *T. viride*). Different incubation times were used because their growth rates were different. After VOC exposure, colony diameter was measured to determine whether each compound affected *Trichoderma* growth. Each *Trichoderma* culture, along with the cellophane membrane used, was placed in a 15 mL tube, frozen using liquid nitrogen, and stored at −80 °C until RNA extraction. Subsequently, a culture plug of *F. oxysporum* f. sp. *lycopersici* strain NRRL54003, which was employed as the tester strain in our previous study (Li et al., 2018), was placed on the medium used to grow *Trichoderma* to measure the extent to which the antifungal molecules secreted by each *Trichoderma* culture inhibit *F. oxysporum* growth. The colony diameter of NRRL54003 was measured after three (for the treatments of

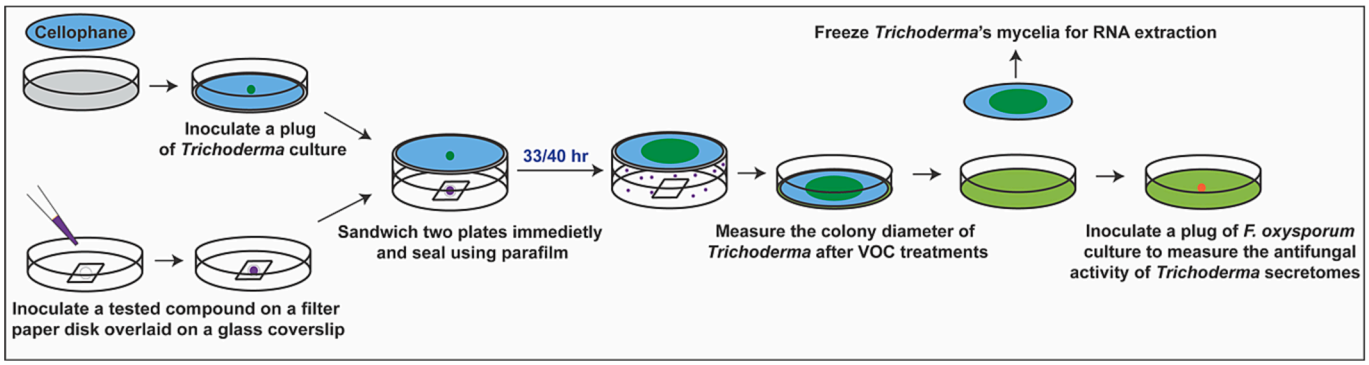


**Fig. 1.** The experimental setup employed to determine the effect of individual VOCs on *Trichoderma* growth and secretion of antifungal molecules. The *Trichoderma* culture, along with the cellophane membrane, was removed after VOC treatment and stored at −80 °C until RNA extraction. Each plate containing antifungal molecules secreted during VOC treatment was inoculated with a plug of *F. oxysporum* culture to measure antifungal activity.

*T. harzianum* and *T. asperellum*) or five (for the treatments of *T. virens* and *T. viride*) days. Isomers of 4-ethylanisole and 3-methyl-1-butanol (Table A.1) were also evaluated. Each treatment consisted of three replicates.

To determine the minimal concentrations of 3-methyl-1-butanol and 4-ethylanisole required to induce antifungal molecule secretion by *Trichoderma*, we conducted the same assay using serially diluted compounds in methanol, and 10 μL of each diluted solution was applied to achieve 5, 10, and 20 μmol/L in the headspace. *Trichoderma* cultures exposed to 10 μL of methanol were used as the control.

### 2.3. Statistical analysis

For all bioassays described above, one-way analysis of variance (ANOVA) was performed using Minitab 18 (Minitab Inc., State College, PA). The significance of the treatments was determined using F-values. When a significant F-test result was observed ($P \leq 0.05$), separation of the means was performed using Fisher's test.

### 2.4. RNA extraction and RNA-seq

Total RNAs were extracted from *Trichoderma* cultures harvested after VOC exposure using the Qiagen RNeasy kit and protocol (Qiagen, Valencia, CA). We also extracted RNAs from *Trichoderma* cultures exposed to whole VOCs released by three *F. oxysporum* strains, NRRL38272 (f. sp. *conglutinans*), 9605 (f. sp. *ciceris*), and NRRL26029 (f. sp. *cubense*), used in our previous study (Li et al., 2018). Three biological replicates were used for this study. RNA purity was evaluated by measuring the $A_{260}/A_{280}$ ratio using a NanoDrop spectrophotometer (Thermo Fisher Scientific). RNA concentration and RNA integrity number (RIN) were measured using an Agilent RNA 6000 Nano Kit and Bioanalyzer 2100 system (Agilent Technologies, CA). Only those with an RIN value above 7.5 were used for cDNA library construction. The cDNA libraries from those exposed to individual compounds and whole *F. oxysporum* VOCs were sequenced using BGISEQ-500 (150-bp paired-end reads; Beijing Genomics Institute) and Illumina HiSeq (150-bp paired-end reads; Novogene), respectively.

### 2.5. RNA-seq data analysis

The resulting raw reads were filtered using SOAPnuke (Chen et al., 2018) to remove low-quality reads (those with more than 20 % of the base qualities lower than 10), reads corresponding to adaptors, and reads with > 5 % unknown bases. The remaining reads were stored as FASTQ files and mapped to the corresponding *Trichoderma* genomes using two RNA-seq aligners, HISAT2 (Hierarchical Indexing for Spliced Alignment of Transcripts 2) (Kim et al., 2015) and STAR (Spliced Transcripts Alignment to a Reference) (Dobin et al., 2013). We used StringTie (Pertea et al., 2015) to reconstruct the transcripts and identify novel transcripts using Cuffcompare, a tool available in Cufflinks (Trapnell et al., 2012). After confirming the protein-coding potential of the novel transcripts using CPC (Kong et al., 2007), they were combined with the transcripts corresponding to previously annotated genes to build a reference transcriptome for read mapping. Clean reads were mapped to the reference transcriptome using Bowtie2 (Langmead and Salzberg, 2012), and transcript levels of individual genes were determined using RSEM (Li and Dewey, 2011) and normalized by FPKM (fragments per kilobase of transcript per million mapped reads) (Trapnell et al., 2010). The Bioconductor package DESeq2 for R statistical analysis (Love et al., 2014) was used to identify differentially expressed genes (DEGs), with an adjusted p-value ≤0.05, and the absolute value of the $Log_2$ fold change ≥1 (at least 2x higher than the expression level in the control) as thresholds. Principal component analysis (PCA) was performed using the DEGseq2 package to visualize the clustering of gene expression patterns between different treatments. Gene expression heatmaps with hierarchical clustering of subsets of DEGs between different treatments were created with the heatmap.2 function in the "gplots" package in R (Warnes et al., 2022). Transcriptomic data were deposited in the Sequence Read Archive (BioProject IDs PRJNA554153 and PRJNA657297-657299). DEGs were subjected to the Gene Ontology (GO) enrichment analysis using GO-MWU (Wright et al., 2015).

### 2.6. Annotation of biocontrol-related genes

The genes for carbohydrate-active enzymes (CAZymes), peptidases, and secondary metabolite biosynthetic enzymes encoded by four *Trichoderma* strains were predicted using dbCAN2 (Zhang et al., 2018), MEROPS release 12.2 (Rawlings et al., 2018), and AntiSMASH 5.1.1 (Blin et al., 2019), respectively. CAZyme annotation was performed in the Anaconda virtual environment using run-dbcan 2.0.11, which includes the annotation tools DIAMOND, HMMER, and Hotpep. Protease annotation was executed via BLASTP 2.2.28+ using the peptidase protein sequences from MEROPS, and each query protein was annotated using the best hit. The AntiSMASH standalone version was run in the Docker environment. SignalP5.0 (Armenteros et al., 2019) was used to determine whether the predicted CAZymes and peptidases contain a signal peptide.

## 3. Results

### 3.1. Identification of F. oxysporum VOCs affecting Trichoderma

We measured *Trichoderma* growth and antifungal molecule secretion after exposure to five candidate VOCs. The assay (Fig. 1) took advantage of the ability of the cellophane membrane to block fungal hyphae, but not secreted metabolites, from reaching the culture medium (Li and

Kang, 2022). All compounds inhibited the growth of the four *Trichoderma* spp. in a concentration-dependent manner (Fig. 2**A**), with 4-ethylanisole being the most inhibitory, followed by 1-hexanol and isopentyl acetate. Growth inhibition by 3-methyl-1-butanol and 2-phenylethanol was significantly lower than that caused by other compounds.

Subsequently, *F. oxysporum* f. sp. *lycopersici* strain NRRL54003 was inoculated onto plates used for culturing *Trichoderma* to determine whether these VOCs affected the secretion of antifungal molecules. We used NRRL54003 because it was used as the tester for the same assay in our earlier study (Li et al., 2018). We observed variations in the growth of NRRL54003 across different VOC treatments, ranging from growth suppression (indicative of increased antifungal molecule secretion) to growth promotion (indicative of suppression of antifungal molecule secretion). NRRL54003 growth was blocked or severely reduced in the media used to treat *T. virens* with 4-ethylanisole and 3-methyl-1-butanol, respectively, at all concentrations (Fig. 2**B**), indicating that both compounds caused *T. virens* to secrete significantly more antifungal molecules. We determined the minimum concentrations of 4-ethylanisole and 3-methyl-1-butanol required for induction. Although both compounds inhibited the growth of *T. virens* at concentrations of 5 and 10 μmol/L (Fig. A.1**A**), they did not significantly increase the secretion of antifungal molecules (Fig. A.1**B**), indicating that the minimum concentrations of 4-ethylanisole and 3-methyl-1-butanol required for induction were between 10 and 20 μmol/L. *T. virens* also secreted more antifungal molecules in response to 500 μmol/L 1-hexanol and 500 μmol/L 2-phenylethanol but not at lower concentrations. The colony diameters of NRRL54003 on the media used for treating *T. virens* with isopentyl acetate were significantly larger than those of the control at all three concentrations, suggesting that isopentyl acetate may function as a suppressor of secretion of antifungal molecules.

In *T. viride,* all compounds, except 4-ethylanisole, induced antifungal molecule secretion in a concentration-dependent manner (Fig. 2**B**). NRRL54003 growth was completely blocked after exposure to 500 μmol/L 1-hexanol and isopentyl acetate. As for 4-ethylanisole, it significantly induced antifungal molecule secretion at 20 and 100 μmol/L but not at 500 μmol/L. Although 1-hexanol, 3-methyl-1-butanol, and 2-phenylethanol caused the induction in *T. harzianum* at all concentrations, the degree of induction was much lower than that observed in *T. virens* and *T. viride* (Fig. 2**B**). In addition, the growth of NRRL54003 was significantly increased following exposure to 100 and 500 μmol/L 4-

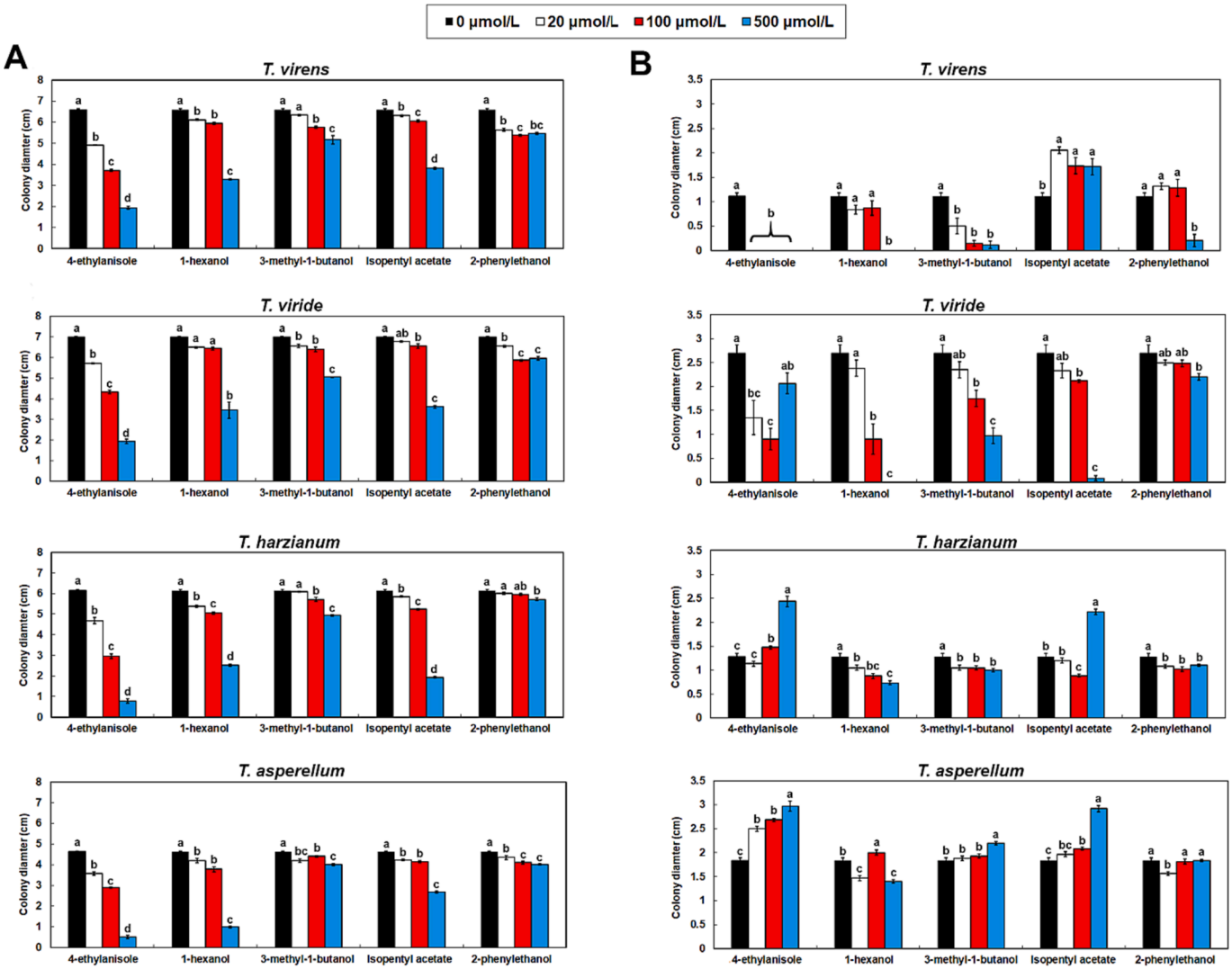


**Fig. 2.** *Trichoderma* growth and secretion of antifungal molecules upon exposure to five VOCs. Each *Trichoderma* culture inoculated on the cellophane membrane overlaid on PDA was exposed to five compounds at three concentrations (20, 100 and 500 μmol/L) for 33 h (*T. harzianum* and *T. asperellum*) or 40 h (*T. virens* and *T. viride*). *Trichoderma* cultured without exposure to VOCs was used as the control for these treatments. After removing the cellophane membrane with *Trichoderma* culture, *F. oxysporum* f. sp. *lycopersici* strain NRRL54003 was inoculated. Colony diameters of (**A**) individual *Trichoderma* spp. after VOC exposure and (**B**) NRRL54003 are shown. The values shown correspond to the mean ± SE of the data from three replicates. Different letters indicate significant differences among treatments based on Fisher's test at $P = 0.05$.

ethylanisole and 500 μmol/L isopentyl acetate. For *T. asperellum*, 20 and 500 μmol/L 1-hexanol and 20 μmol/L 2-phenylethanol caused induction, whereas the other treatments either had no effect or appeared to suppress the secretion of antifungal molecules (Fig. 2**B**).

We evaluated the effect of the structural isomers of 4-ethylanisole and 3-methyl-1-butanol on *T. virens* to test whether compound-specific receptors are likely to be involved in the recognition of VOCs. All isomers inhibited the growth of *T. virens* in a concentration-dependent manner (Fig. A.2**A**) and caused *T. virens* to secrete more antifungal molecules at least at two concentrations. However, the degree of

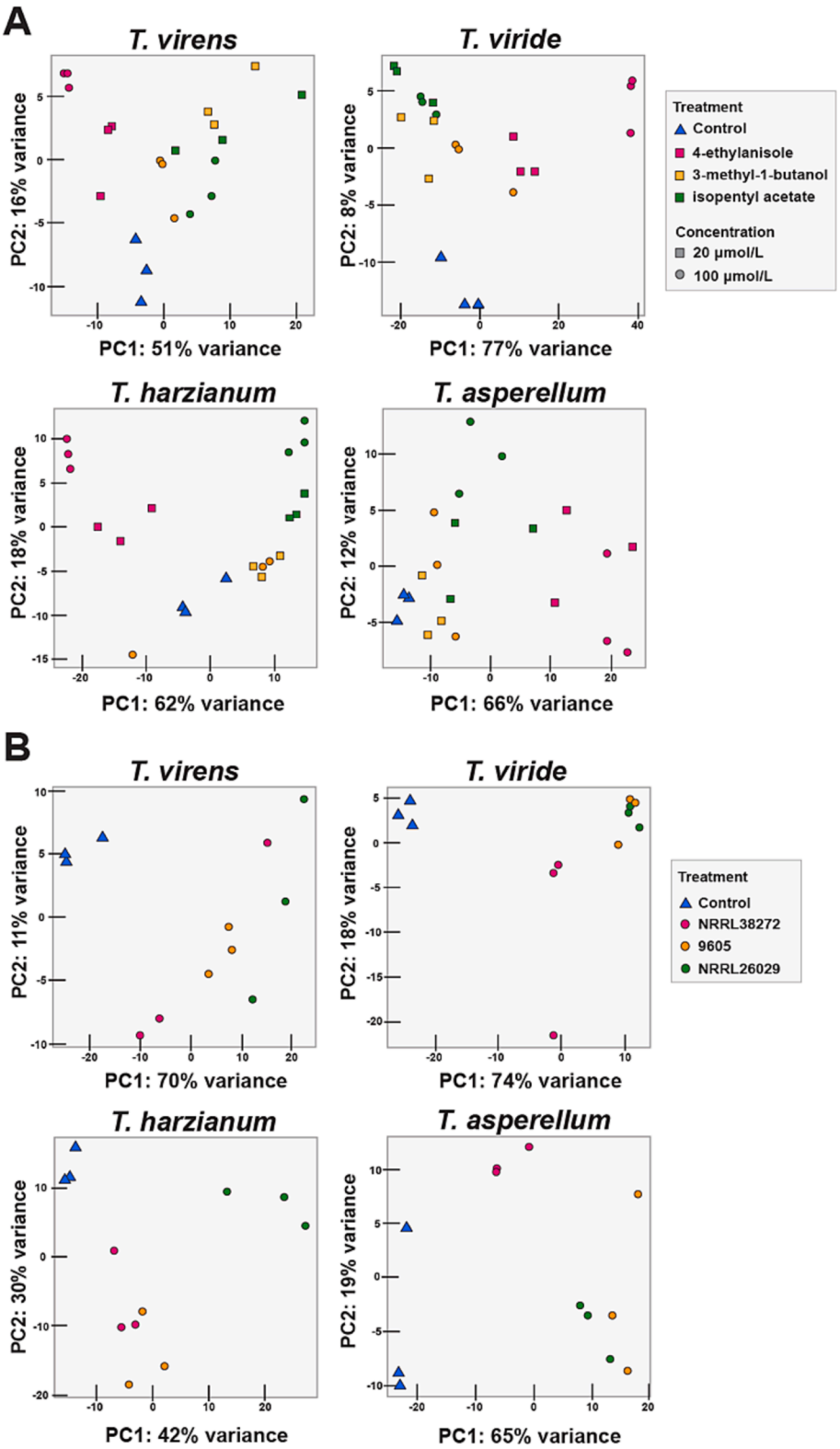


**Fig. 3.** Principal component analysis (PCA) of gene expression patterns. Data from *T. virens, T. viride, T. harzianum,* and *T. asperellum* exposed to (**A**) three individual compounds at two different concentrations and (**B**) VOCs produced by the three *F. oxysporum* strains were analyzed. Three biological replicates were used for each treatment.

induction varied among the isomers (Fig. A.2**B**).

### 3.2. Transcriptome analysis after exposure to individual compounds and VOCs produced by F. oxysporum cultures revealed many differentially expressed genes (DEGs)

We analyzed the transcriptomes of four *Trichoderma* spp. after exposure to 4-ethylanisole, 3-methyl-1-butanol, and isopentyl acetate (20 and 100 µmol/L). *Trichoderma* spp. exposed to VOCs produced by *F. oxysporum* strains NRRL38272 (f. sp. *conglutinans*), 9605 (f. sp. *ciceris*), and NRRL26029 (f. sp. *cubense*) were also analyzed. These strains were chosen because their VOCs highly induced antifungal molecule secretion by *T. virens* and *T. viride* (Li et al., 2018). In contrast, *T. asperellum* did not respond to their VOCs, and *T. harzianum* only responded to VOCs from NRRL26029 (Li et al., 2018).

Two alignment methods, HISAT2 and STAR, were compared to determine which one provided a higher rate of transcript alignment to the reference genomes (Table A.2). STAR consistently provided higher alignment rates (~98.97 % compared to ~ 95.33 % by HISAT2), thus it was used as the aligner for all the RNA-seq data. We detected transcripts from 85.40 %, 84.68 %, 82.26 %, and 89.29 % of the predicted protein-coding genes in *T. virens, T. viride, T. harzianum*, and *T. asperellum*, respectively (Table A.3).

#### 3.2.1. Principal component analysis (PCA)

We first performed PCA to determine the effects of different VOC treatments on the gene expression profiles. Separate PCA plots were generated for individual compound treatments (Fig. 3**A**) and those treated with VOCs produced by *F. oxysporum* cultures (Fig. 3**B**) to eliminate potential batch effects. Distinct differences were observed between most VOC-treated samples and controls, suggesting that VOC treatment significantly altered gene expression in all *Trichoderma* spp. One exemption was *T. asperellum* exposed to 3-methyl-1-butanol (no clear separation between the 3-methyl-1-butanol treatment and the control). Samples treated with 3-methyl-1-butanol and isopentyl acetate, although causing opposite effects on antifungal molecule secretion in *T. virens* and *T. viride*, tended to cluster together and separate from those exposed to 4-ethylanisole, suggesting that transcriptome changes caused by the former two are distinct from those caused by the latter. When *T. virens* was exposed to VOCs from *F. oxysporum* cultures, the samples were widely dispersed in the plot, with no clear grouping patterns, indicating distinct transcriptome responses to VOCs produced by different *F. oxysporum* strains. In contrast, the samples clustered well based on their effects on antifungal molecule secretion in *T. harzianum* and *T. viride* (Fig. 2**B**). Although none of the VOC treatments induced antifungal molecule secretion by *T. asperellum* (Li et al., 2018), significant differences were found between treatments with VOCs from NNRL38272 and those treated with VOCs from 9605 and NRRL26029 (Fig. 2**B**).

#### 3.2.2. Patterns of DEGs

The expression of hundreds to more than 1,000 genes in all species was significantly affected by the treatments (Fig. 4). The only exception was *T. asperellum* treated with 3-methyl-1-butanol (<100 DEGs). The identities of all DEGs in the four *Trichoderma* spp. are shown in Tables A.4-A.7. Among individual compound treatments (Fig. 4**A**), 100 µmol/L 4-ethylanisole resulted in a much greater number of DEGs than 20 µmol/L 4-ethylanisole did in all *Trichoderma* spp. In contrast, all species, except *T. asperellum,* treated with 20 µmol/L 3-methyl-1-butanol had more DEGs than those treated with 100 µmol/L 3-methyl-1-butanol. More DEGs were found in *T. virens* and *T. viride* following exposure to 20 µmol/L isopentyl acetate while *T. harzianum* and *T. asperellum* had more DEGs after exposure to 100 µmol/L isopentyl acetate. VOCs from *F. oxysporum* cultures affected more genes than individual compounds: as many as 1,228, 1,426, and 1,412 DEGs were detected in *T. virens, T. viride, and T. harzianum*, respectively, after exposure to VOCs produced by NRRL26029, and VOCs from 9605 resulted in the largest number of DEGs in *T. asperellum* with a total of 1,253 DEGs (Fig. 4**C**).

We looked at DEGs that were shared a) between two concentrations of each compound (Fig. 4**A**), b) among the three compounds (Fig. 4**B**), and c) among VOCs from three *F. oxysporum* strains (Fig. 4**C**) and found the following patterns. As for 4-ethylanisole, more down-regulated DEGs than up-regulated DEGs were shared between the two concentrations in all *Trichoderma* spp., except *T. harzianum* (Fig. 4**A**). In contrast, 3-methyl-1-butanol and isopentyl acetate resulted in more up-regulated DEGs than down-regulated DEGs shared between the two concentrations. Not surprisingly, significantly fewer shared DEGs were found among the samples treated with the three compounds than those shared between 20 and 100 µmol/L of each compound (Fig. 4**A** and **B**). Among the DEGs shared by all culture-derived VOC treatments, the numbers of down-regulated genes were higher than those up-regulated (Fig. 4**C**). In addition, there was a lot of overlap in DEGs between NRRL26029 and 9605 treatments in all *Trichoderma* spp., except for up-regulated DEGs in *T. harzianum* (more shared between NRRL38272 and 9605).

#### 3.2.3. Gene ontology (GO) enrichment analysis

This analysis was performed to identify cellular processes that were significantly affected by VOCs. The GO terms enriched in individual

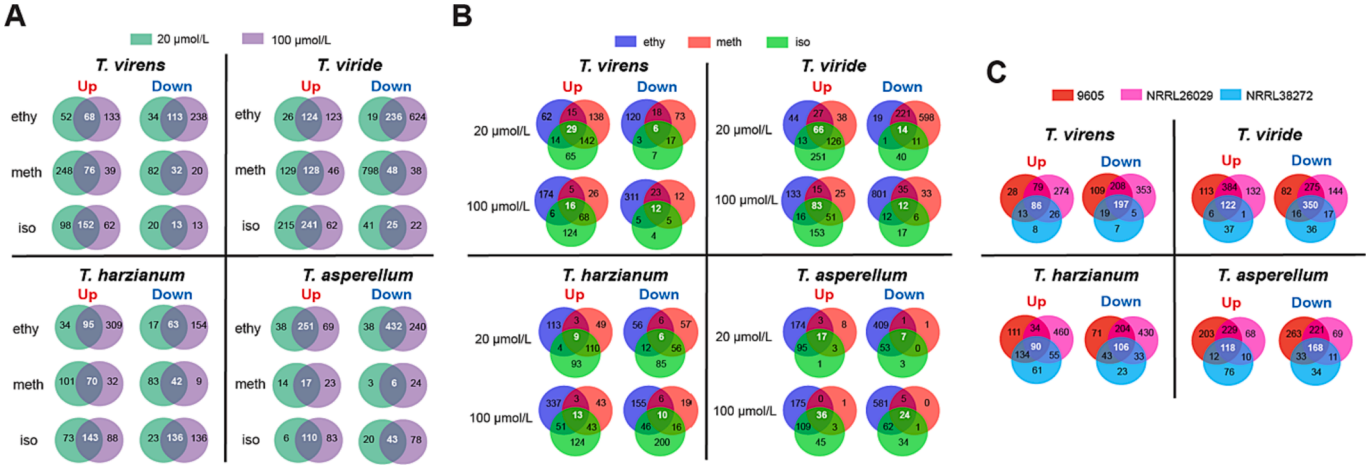


**Fig. 4.** Venn diagrams showing similarly regulated genes in the *Trichoderma* spp. subjected to different VOC treatments. Commonly up-regulated and down-regulated genes in individual species after exposure to (**A**) two concentrations (20 µmol/L and 100 µmol/L) of 4-ethylanisole (ethy), 3-methyl-1-butanol (meth), and isopentyl acetate (iso), (**B**) three compounds at 20 µmol/L and 100 µmol/L, and (**C**) VOCs produced by the three *F. oxysporum* strains are presented.

species differed significantly even when they were treated with the same compound (Fig. 5). The DEGs that were affected in more than one species participate in a variety of biological processes (Fig. 5 and Figs. A.3-A.6), such as synthesizing/metabolizing various types of organic compounds, ion/carbohydrate transport, proteolysis, response to stimulus, signal transduction, chromosome organization, RNA processing, and DNA metabolism (Fig. 5). In contrast, 28 GO terms were enriched in only one species.

In *T. virens*, the GO terms involved in proteolysis and organonitrogen compound biosynthesis were significantly enriched after multiple treatments, including two concentrations of 4-ethylanisole and whole VOCs from three *F. oxysporum* strains (Fig. 5), but different expression patterns were observed: a) DEGs involved in proteolysis were up-regulated by 4-ethylanisole and down-regulated by whole VOCs and b) DEGs involved in organonitrogen compound biosynthesis were down-regulated by 4-ethylanisole and up-regulated by whole VOCs (Fig. A.3). The organonitrogen compound biosynthetic process was enriched in *T. viride* after all treatments, followed by proteolysis (Fig. 5), with 4-ethylanisole causing the reverse pattern of expression compared to the other VOC treatments (Fig. A.4). Only a few GO terms were shared between the two concentrations of the same compound in *T. harzianum*, and none was shared among the three *F. oxysporum* strains (Fig. 5 and Fig. A.5). In *T. asperellum*, organic acid metabolism was enriched after all VOC treatments (Fig. 5) with individual compounds and VOCs from the three *F. oxysporum* strains causing their down-regulation and up-regulation, respectively (Fig. A.6). Those associated with response to stimulus were enriched in *T. asperellum* after individual compound treatments (Fig. A.6).

We hypothesized that the significant growth inhibition of all *Trichoderma* spp. by individual compounds (Fig. 2**A**) may be due to cellular stress. Genes involved in the response to oxidative stress were minimally affected by most treatments (Fig. 5 and Figs. A.3-A.6), but those involved in programmed cell death or apoptotic process were significantly up-regulated (padj = 0.006) in *T. harzianum* following exposure to 100 μmol/L 4-ethylanisole (Fig. 5 and Fig. A.5).

### 3.3. Different treatments affected biocontrol-related genes in distinct ways

GO enrichment analysis showed that various biological functions likely associated with biocontrol were differentially regulated by VOCs, which led to the hypothesis that *Trichoderma* BCAs regulate their biocontrol machinery in response to specific VOCs produced by neighboring fungi. We analyzed the relative gene expression levels of the DEGs in the following functional categories: fungal cell wall degradation, protein hydrolysis, and secondary (specialized) metabolism (see Tables A.8-A.11 for the average fold-change of individual DEGs in each category).

#### 3.3.1. Fungal cell wall degradation

Most enzymes involved in fungal cell wall degradation belong to six glycoside hydrolase (GH) families: GH18 chitinase, GH17, GH55, GH64, GH81 β-glucanase, and GH75 chitosanase (Kubicek et al., 2011; Schmoll et al., 2016). Collectively, 16, 17, 25, and 18 genes belonging to these GH families were differentially expressed in *T. virens, T. viride, T. harzianum,* and *T. asperellum*, respectively (Tables A.8-A.11). The expression of chitinase genes appeared to be widely affected by many VOC treatments, followed by β-glucanase. While most of the VOC treatments affected their expression in both ways, we found a few treatments that only caused up- or down-regulation of these genes depending on the species (Table 1).

#### 3.3.2. Protein hydrolysis

Some proteases and peptidases secreted by *Trichoderma* antagonize other microbes (Hyder et al., 2017). In response to VOC treatment, 41, 67, 44, and 51 peptidase genes were differentially expressed in *T. virens*, *T. viride*, *T. harzianum*, and *T. asperellum*, respectively (Figs. A.7-A.10 and Tables A.8-A.11). In addition to well-characterized peptidase genes, VOC treatment also affected the expression of two groups of genes: a) unassigned peptidases (putative peptidases containing all the catalytic residues but could not be assigned to specific subfamilies because of the lack of sequence similarity to known holotypes) and b) those homologous to a characterized peptidase family but lacking one or more of the expected catalytic residues. In *T. virens,* all treatments except 100 μmol/L 4-ethylanisole caused more genes up-regulated than those down-regulated (Fig. A.7). Four genes, including polyporopepsin (an aspartic peptidase) and three uncharacterized peptidases, were up-regulated by both 3-methyl-1-butanol and isopentyl acetate. Five genes encoding grifolisin (a serine proteinase), carboxypeptidase A (a zinc metalloprotease), polyporopepsin, aorsin (a serine proteinase), and scytalidoglutamic peptidase (a glutamic protease) were commonly activated by VOCs from the three *F. oxysporum* strains (Fig. A.7); the latter three were also induced by the same treatments in *T. viride* (Fig. A.8). In *T. viride,* 100 μmol/L 4-ethylanisole had the greatest effect on peptidase gene expression, down-regulating 37 genes, whereas the other compounds affected the expression of less than ten genes. Eleven and 13 genes were commonly up- and down-regulated, respectively, by VOCs produced by NRRL26029 and 9605, with a subset of them being affected by VOCs from NRRL38272 as well (Fig. A.8). In *T. harzianum*, VOCs from NRRL26026 affected the highest number of peptidase genes, whereas 3-methyl-1-butanol affected the fewest genes (Fig. A.9). In *T. asperellum*, 4-ethylanisole and isopentyl acetate activated the same ten genes, including a Prb1 peptidase (a serine proteinase), a fungalysin (a metalloprotease), a PoSI-type peptidase (a serine proteinase), and seven unassigned peptidases and non-peptidase homologs (Fig. A.10).

#### 3.3.3. Secondary metabolism

The VOC treatments affected the expression of 12, 17, 14, and 24 non-ribosomal peptide synthetase (NRPS), polyketide synthase (PKS), and terpene cyclase genes in *T. virens*, *T. viride*, *T. harzianum*, and *T. asperellum*, respectively (Fig. 6 and Tables A.8-A.11**)**. In *T. virens*, at least two compounds induced five NRPS/NRPS-like genes, one Type I PKS gene, and one terpene cyclase gene (Fig. 6**A**). Compared to the individual compounds, only one gene was up-regulated and two genes were down-regulated by VOCs produced by the three *F. oxysporum* strains. *T. viride* increased the expression of four NRPS/NRPS-like genes and one Type I PKS gene (Fig. 6**B**), with the NRPS genes OSK-36_GLEAN_10001439 and OSK-36_GLEAN_10001440 being up-regulated by all three compounds at both concentrations. Culture-derived VOCs tended to activate the expression of Type I PKS genes while suppressing the expression of NRPS/NRPS-like genes. In *T. harzianum*, VOCs produced by NRRL26029 induced the largest number of genes, followed by 4-ethylanisole (Fig. 6**C**). In *T. asperellum,* 4-ethylanisole and isopentyl acetate induced the same four NRPS/NRPS-like and five Type I PKS genes, but 4-ethylanisole also suppressed nine other genes (Fig. 6**D**).

Two VOC-induced NRPS genes in *T. virens*, OSK-13_GLEAN_10001469 and OSK-13_GLEAN_10001470, are located in the same gene cluster (Fig. 7**A**). OSK-13_GLEAN_10001469 exhibited 77 % nucleotide identity to *T. gamsii* surfactin synthetase subunit 3, but the function of OSK-13_GLEAN_10001470 remains unknown. The cluster harbors eight accessory enzyme genes and two genes encoding transporters, four of which were induced by individual compound treatments. For example, OSK-13_GLEAN_10001471, OSK-13_GLEAN_10001472, and OSK-13_GLEAN_10001473, which are predicted to encode acyl-CoA N-acyltransferase, siderophore iron transporter, and pyridine nucleotide-disulfide oxidoreductase, respectively, were up-regulated by 20 μmol/L isopentyl acetate. In contrast, VOCs produced by NRRL26029 and 9605 suppressed five and two genes, respectively, in the cluster. We found a gene cluster in *T. viride* that was homologous to the *T. virens* NRPS gene cluster. In *T. viride*, the two NRPS genes and two genes encoding acyl-CoA N-acyltransferase and pyridine nucleotide-disulfide oxidoreductase, respectively, were up-regulated by

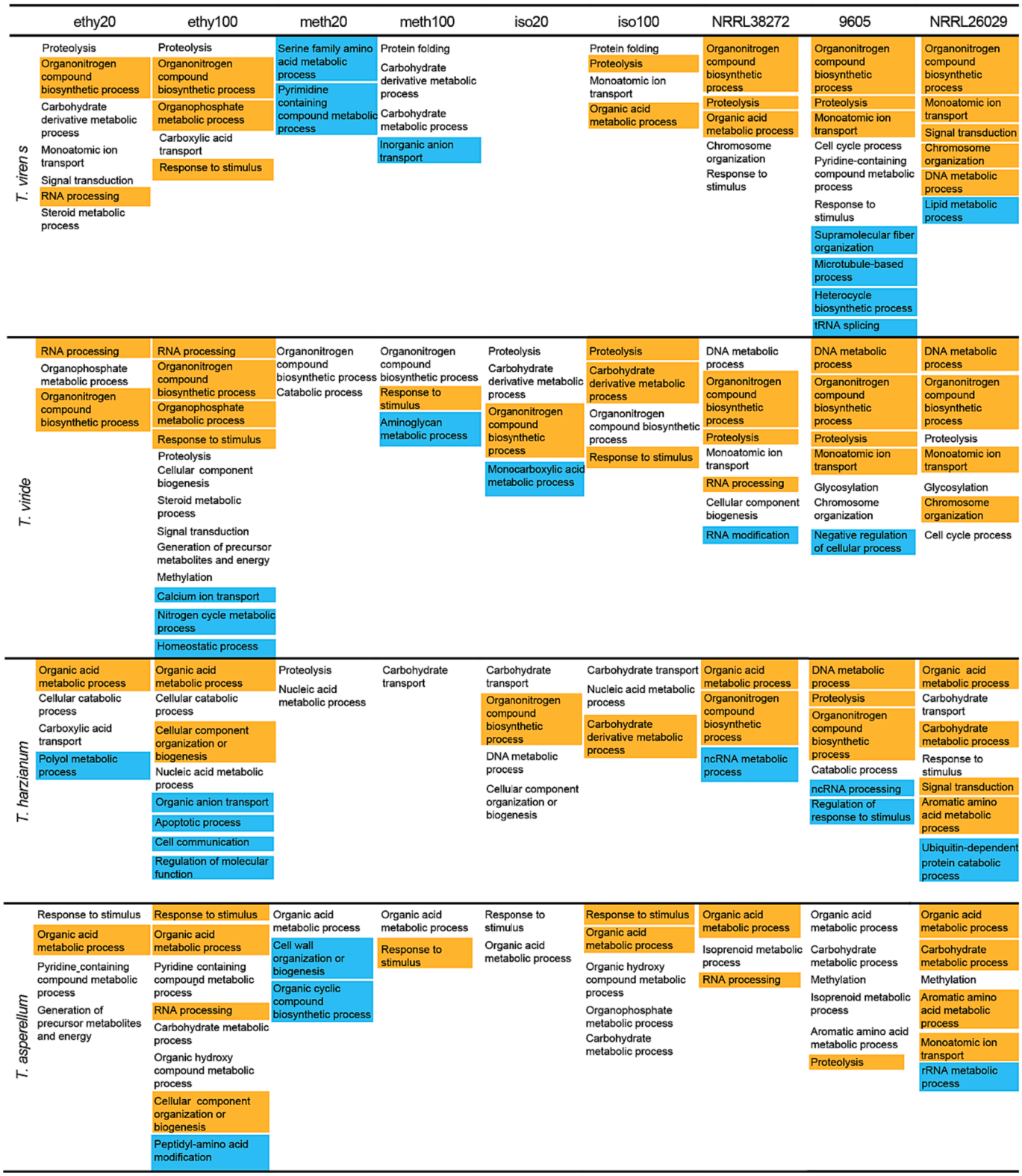


**Fig. 5.** Gene ontology (GO) terms enriched (adjusted p-value $\leq 0.05$) among the DEGs in four *Trichoderma* spp. after exposure to 4-ethylanisole (ethy), 3-methyl-1-butanol (meth), isopentyl acetate (iso), and whole VOCs produced by the three *F. oxysporum* strains are shown. The numbers 20 and 100 indicate 20 μmol/L and 100 μmol/L, respectively. For each VOC treatment, GO terms enriched in two or more species are marked in orange. The unique GO terms enriched in one species by one treatment are highlighted in blue.

**Table 1**
Fungal cell wall degradation-associated genes differentially expressed in response to individual VOC treatments.

| | *T. virens*[2] | | | | *T. viride*[2] | | | | *T. harzianum*[2] | | | | *T. asperellum*[2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Treatment**[1] | **Cht** | **Glc** | **Chs** | **Total** | **Cht** | **Glc** | **Chs** | **Total** | **Cht** | **Glc** | **Chs** | **Total** | **Cht** | **Glc** | **Chs** | **Total** |
| **ethy20** | 1 | – | 1 | 2 | 3 | 1 | 0 | 4 | 1/1 | 2 | 0 | 1/3 | 4/1 | 1/2 | 0 | 5/3 |
| **ethy100** | 5 | 2 | 3 | 10 | 2/1 | 2/1 | 0 | 4/2 | 1 | 4 | 0 | 5 | 4/2 | 1/2 | 0 | 5/4 |
| **meth20** | 1 | 1 | 1 | 3 | 1 | 0 | 0 | 1 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| **meth100** | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1/1 | 2 | 0 | 0 | 2 | 0 | 0 | 0 | 0 |
| **iso20** | 0 | 0 | 0 | 0 | 1/2 | 1 | 0 | 1/3 | 1/2 | 1/3 | 3 | 2/8 | 1 | 0 | 0 | 1 |
| **iso100** | 0 | 0 | 0 | 0 | 1/1 | 1 | 0 | 1/2 | 1/6 | 3 | 4 | 1/13 | 1/1 | 0 | 1 | 2/1 |
| **NRRL38272** | 0 | 1 | 1 | 2 | 3 | 1/1 | 1 | 4/2 | 8 | 1/1 | 1 | 1/10 | 4/2 | 0 | 1 | 4/3 |
| **9605** | 2 | 1 | 0 | 2/1 | 3/2 | 3/1 | 1/1 | 7/4 | 2 | 1/2 | 1 | 1/5 | 2/5 | 1/2 | 1 | 3/8 |
| **NRRL26029** | 4/1 | 1/1 | 1 | 5/3 | 2/2 | 3/1 | 1/2 | 6/5 | 9 | 1/4 | 2 | 1/15 | 2/6 | 1 | 1 | 3/7 |

1. The treatments included 4-ethylanisole (ethy), 3-methyl-1-butanol (meth), isopentyl acetate (iso), and VOCs produced by the three *F. oxysporum* strains. The numbers 20 and 100 indicate 20 μmol/L and 100 μmol/L, respectively.
2. DEGs (red = up-regulated and blue = down-regulated) encoding chitinase (Cht), β-glucanase (Glc), and chitosanase (Chs).

all three compounds at both concentrations, whereas these genes and the ABC multidrug transporter gene were suppressed by VOCs produced by 9605 and NRRL26029 (Fig. 7**A**).

In *T. virens*, a terpene cyclase (OSK-13_GLEAN_10008488) and some genes encoding cytochrome P450 monooxygenases in a terpene biosynthetic gene cluster were up-regulated by 3-methyl-1-butanol and isopentyl acetate (Fig. 7**B**). However, these genes were significantly suppressed by 100 μmol/L 4-ethylanisole. None of the genes in the cluster was affected by 20 μmol/L 4-ethylanisole or VOCs produced by *F. oxysporum* strains. The terpene cyclase gene showed 98.67 % nucleotide sequence identity with the *vir4* gene in *T. virens*, which was suggested to be involved in the production of monoterpenes and sesquiterpenes (Crutcher et al., 2013).

## 4. Discussion

*Trichoderma* has been widely used to control diverse pathogens including *F. oxysporum* (Druzhinina et al., 2011; Harman et al., 2004; Hyder et al., 2017; Sivan and Chet, 1989; Zhang et al., 2014). One strategy employed by *Trichoderma,* as well as other microbial BCAs, for pathogen suppression is to secrete antimicrobial metabolites, including VOCs (Kang et al., 2021). Antibiosis is not the only function of metabolites secreted by BCAs. Zhang et al., (2022a) found that 6-methyl-2-heptanone, a VOC emitted by *Bacillus subtilis* ZD01, affects multiple processes in the early blight pathogen, *Alternaria solani*. Pathogens and diverse soil microbes also release bioactive metabolites; however, how such metabolites affect BCAs remains unclear. Considering that BCAs interact with diverse microbes, not just target pathogens, understanding how metabolite-mediated microbial interactions affect BCAs is crucial to making biocontrol an effective and reliable pesticide alternative. Here, we characterized how five VOCs that are commonly released by diverse *F. oxysporum* strains (Li et al., 2018) affect the growth of four *Trichoderma* spp. and their secretion of antifungal molecules (Fig. 2) and how individual *Trichoderma* spp. modulate gene expression (Figs. 3-6 and Figs. A.3-A.6) in response to three specific VOCs and whole VOCs produced by three *F. oxysporum* strains.

Consistent with our previous study showing the inhibition of *Trichoderma* growth by *F. oxysporum* VOCs (Li et al., 2018), the five compounds tested suppressed *Trichoderma* growth to varying degrees, with 4-ethylanisole causing the highest inhibition and 2-phenylethanol being the least inhibitory (Fig. 2**A**). The degree of growth inhibition by each compound was similar among the four *Trichoderma* spp., suggesting that each compound likely disrupts some growth-related processes conserved among these species. This is the first report of 4-ethylanisole acting as an antifungal molecule (Fig. 2**A** and Fig. A.1**A**). Although the production of 4-ethylanisole by other fungal species remains to be analyzed, considering that its synthesis occurs via the phenylalanine biosynthesis pathway, other fungi likely produce this compound. Isopentyl acetate, 1-hexanol, 3-methyl-1-butanol, and 2-phenylethanol are produced by many bacteria and fungi that suppress other microbes (Dalilla et al., 2015; Kyoui et al., 2023; Masoud et al., 2005; Wonglom et al., 2020; Zhang et al., 2019). The VOCs produced by individual *F. oxysporum* strains include additional compounds that have been shown to inhibit other microbes but are not produced by all strains analyzed (Li et al., 2018), suggesting the involvement of multiple VOCs in suppressing *Trichoderma* growth. Previous studies have shown that antifungal VOCs are produced by both *Trichoderma* (Amin et al., 2010; Li et al., 2018; Meena et al., 2017; Wonglom et al., 2020) and *F. oxysporum* (Li et al., 2018), suggesting that the production of antifungal VOCs is a common strategy employed by fungi to suppress other fungi.

*Trichoderma* BCAs can sense other fungi by recognizing small molecules associated with them (Atanasova et al., 2013; Druzhinina et al., 2011; Seidl et al., 2009). However, it remains unclear whether they recognize specific VOCs produced by other fungi. Our previous work suggested the potential role of unknown *F. oxysporum* VOC(s) as signal (s) that some *Trichoderma* spp. recognize to increase antifungal molecule secretion (Li et al., 2018). To test this hypothesis, we determined whether the five compounds commonly produced by multiple *F. oxysporum* strains significantly affected the secretion of antifungal molecules by *Trichoderma* spp. (Fig. 1). In contrast to the similar growth responses of these species to each compound (Fig. 2**A**), the secretion of antifungal molecules appeared to vary significantly (Fig. 2**B**), suggesting that each species differently regulates the latter response. Overall, antifungal molecule secretion by *T. virens* and *T. viride* was highly induced by most compounds while that by *T. harzianum* and *T. asperellum* was less affected or not changed. This pattern is similar to their responses to whole VOCs produced by diverse *F. oxysporum* strains (Li et al., 2018): *T. virens* and *T. viride* markedly increased antifungal molecule secretion upon exposure to VOCs produced by all *F. oxysporum* strains; *T. harzianum* moderately increased the secretion in response to VOCs from only some strains; and *T. asperellum* did not respond. Exposure to certain VOCs seemed to suppress the secretion of antifungal molecules by some *Trichoderma* spp., including 4-ethylanisole to *T. asperellum* and isopentyl acetate to *T. virens* and *T. asperellum* (Fig. 2**B**). These findings suggest that *F. oxysporum* may use some VOCs to weaken *Trichoderma*'s chemical attack, which is reminiscent of the pathogen effectors in plants (Zhang et al., 2022b).

Our study raises several questions, including why the secretion of antifungal molecules varies among species in response to VOCs. Identification of the antifungal metabolite(s) differentially regulated by *F. oxysporum* VOCs should help to address this question. Our earlier study (Li et al., 2018) showed that *T. harzianum* likely increased the secretion of anthraquinone(s), which are chromogenic polyketides exhibiting antimicrobial activity (Liu et al., 2009), in response to VOCs produced by some *F. oxysporum* strains. However, the patterns of their production did not correlate with the degree of antifungal activities observed (Li et al., 2018), and no chromogenic metabolites seemed to be produced by the other *Trichoderma* spp. These observations suggest that

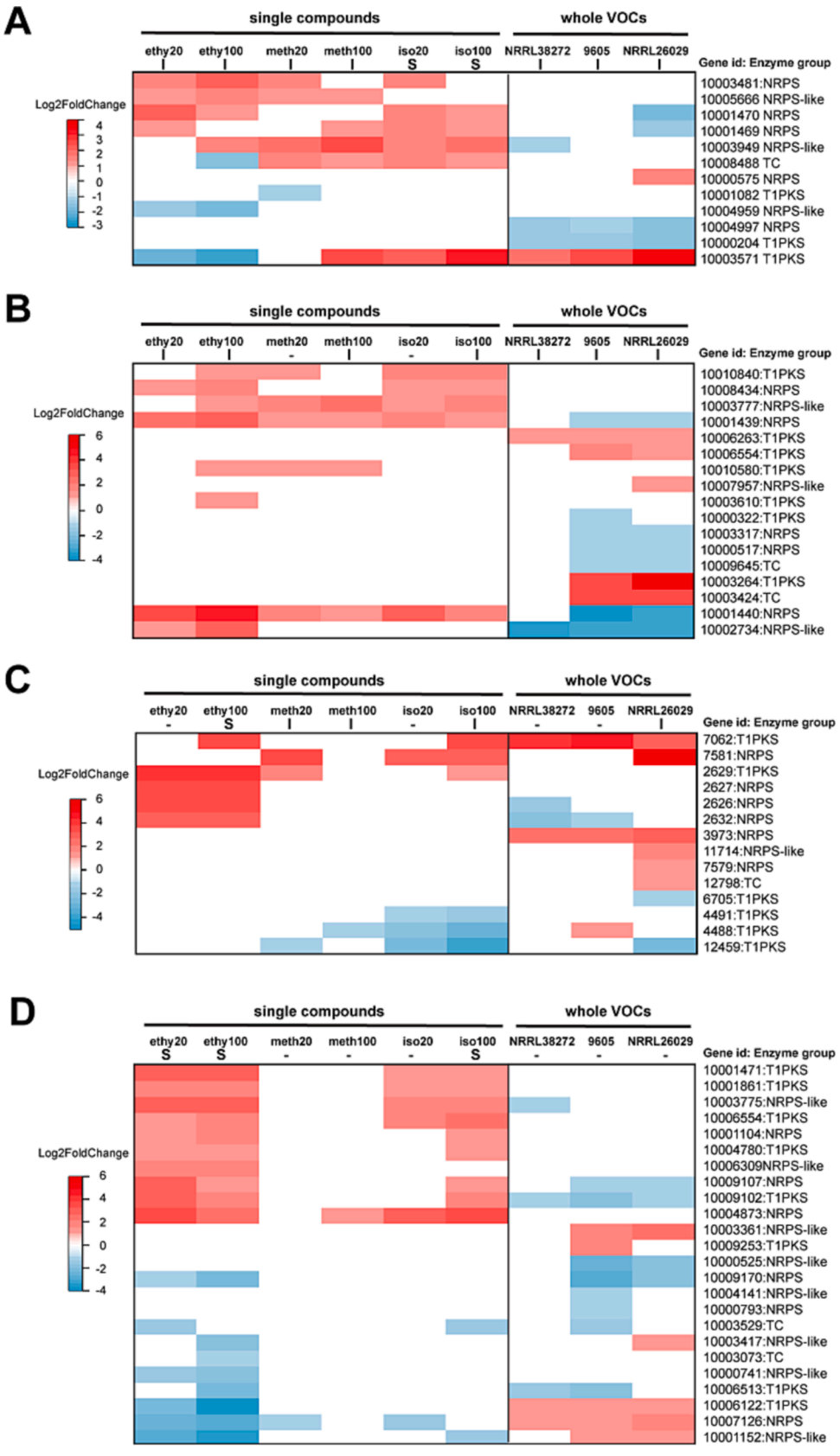


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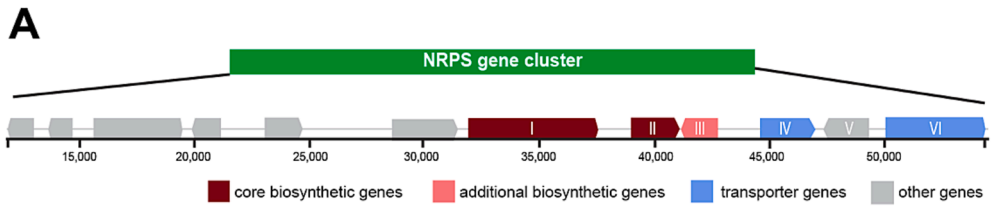
**Fig. 6.** Heatmap of secondary metabolism genes differentially expressed in response to 4-ethylanisole (ethy), 3-methyl-1-butanol (meth), isopentyl acetate (iso), and VOCs produced by three *F. oxysporum* strains. Relative transcription levels of the NRPS (non-ribosomal peptide synthetase)/NRPS-like, T1PKS (Type I polyketide synthase), and TC (terpene cyclase) genes in (**A**) *T. virens,* (**B**) *T. viride,* (**C**) *T. harzianum*, and (**D**) *T. asperellum* are shown. Each cell represents the average fold-change in three biological replicates in response to each VOC treatment compared to the control (see Tables A.8-A.11 for details). The numbers 20 and 100 indicate 20 μmol/L and 100 μmol/L, respectively. White = no significant difference (padj > 0.05), red = significantly up-regulated, blue = significantly down-regulated (padj ≤ 0.05). I and S beneath individual treatments indicate the induction and suppression of antifungal molecule secretion, respectively.

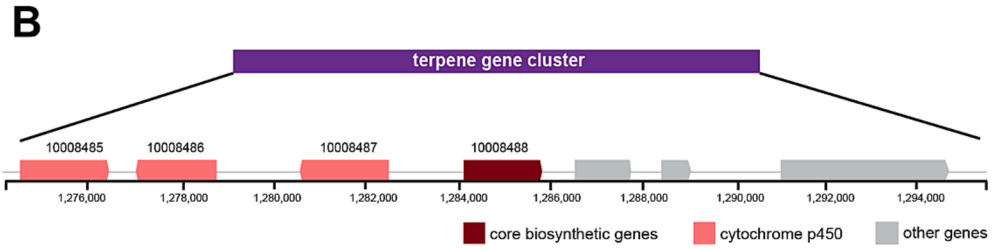



**Relative transcription levels in *T. virens***

| Gene ID | ethy20 | ethy100 | meth20 | meth100 | iso20 | iso100 | NRRL38279 | 9605 | NRRL26029 |
|---|---|---|---|---|---|---|---|---|---|
| 10001469 (I) | 1.28 | – | – | 1.02 | 1.41 | 1.06 | – | – | -1.33 |
| 10001470 (II) | 2.04 | 1.03 | – | – | 1.46 | 1.16 | – | – | -1.83 |
| 10001471 (III) | 1.46 | – | – | – | 1.02 | – | – | -1.08 | -2.10 |
| 10001472 (IV) | 1.07 | – | 1.37 | 1.01 | 1.17 | – | – | -1.45 | – |
| 10001473 (V) | 1.53 | – | – | 1.14 | 1.23 | 1.44 | – | – | -1.76 |
| 10001474 (VI) | 1.34 | – | – | – | – | 1.14 | – | – | -1.45 |

**Relative transcription levels in *T. viride***

| Gene ID | ethy20 | ethy100 | meth20 | meth100 | iso20 | iso100 | NRRL38279 | 9605 | NRRL26029 |
|---|---|---|---|---|---|---|---|---|---|
| 10001439 (I) | 2.35 | 2.78 | 1.07 | 1.14 | 1.97 | 1.55 | – | -1.31 | -1.02 |
| 10001440 (II) | 3.65 | 4.41 | 1.66 | 1.53 | 2.74 | 1.98 | – | -3.68 | -3.08 |
| 10001441(III) | 2.63 | 3.30 | 3.30 | 1.14 | 2.23 | 1.72 | – | -4.06 | -3.20 |
| 10001442 (IV) | 1.03 | 1.04 | 1.04 | – | – | – | – | -1.14 | – |
| 10001443 (V) | 2.24 | 2.72 | 2.72 | 1.04 | 1.78 | 1.52 | – | -2.59 | -1.68 |
| 10001444 (VI) | 1.95 | 2.37 | 2.37 | – | 1.60 | 1.30 | – | -1.82 | -1.46 |




**Relative transcription levels in *T. virens***

| Gene ID | ethy20 | ethy100 | meth20 | meth100 | iso20 | iso100 | NRRL38279 | 9605 | NRRL26029 |
|---|---|---|---|---|---|---|---|---|---|
| 10008485 | – | -1.27 | 1.54 | 1.18 | 1.33 | 1.07 | – | – | – |
| 10008486 | – | -1.41 | 1.28 | 1.01 | 1.09 | – | – | – | – |
| 10008487 | – | -1.40 | 1.26 | – | – | – | – | – | – |
| 10008488 | – | -1.49 | 1.58 | 1.23 | 1.36 | 1.12 | – | – | – |

**Fig. 7.** Effects of individual VOC treatments on gene expression in two specific secondary metabolism gene clusters. Expression patterns of the genes in (**A**) a non-ribosomal peptide synthetase (NRPS) gene cluster in *T. virens* and *T. viride* and (**B**) a terpene gene cluster in *T. virens* are shown. The genome organization of the clusters and relative transcription levels of the genes in this cluster in response to 4-ethylanisole (ethy), 3-methyl-1-butanol (meth), isopentyl acetate (iso), and VOCs produced by the three *F. oxysporum* strains compared to the corresponding controls are shown. The numbers 20 and 100 indicate 20 μmol/L and 100 μmol/L, respectively. The values correspond to the average $Log_2$ fold change in three biological replicates. No significant differences were marked using -.

different antifungal metabolites are involved in the suppression *of F. oxysporum.* Our preliminary LC-MS analysis of secreted *T. virens* metabolites after exposure to *F. oxysporum* VOCs revealed extensive changes in secreted metabolites, indicating that a comparative metabolomic analysis, coupled with an antifungal activity assay, is needed to identify candidate antifungal metabolite(s).

RNA-seq analysis was performed to help predict candidate antifungal metabolite(s) by determining which genes and biological functions are differentially regulated by *F. oxysporum* VOCs and to help test the hypothesis that *Trichoderma* increases the production of biocontrol-associated proteins and metabolites by recognizing specific pathogen VOCs. Multiple factors (compound, concentration, *F. oxysporum* strain, and *Trichoderma* species) appear to influence the genes that are differentially regulated. Because we exposed *Trichoderma* spp. to individual compounds and mixtures of VOCs for only one duration (33 h for *T. harzianum* and *T. asperellum* and 40 h for *T. virens* and *T. viride*) before the RNA-seq analysis, resulting DEGs likely consist of mixtures of initial and subsequent transcriptional responses. Accordingly, our current

dataset is not sufficient to make strong inferences about the potential mechanisms of VOC-mediated manipulation of *Trichoderma* BCAs by *F. oxysporum* VOCs and their significance in biocontrol. With these limitations in mind, we discuss some notable patterns and future questions.

Extensive changes in gene expression upon exposure to individual compounds and culture-derived VOCs (Table 1, Figs. 3-6 and Figs. A.3-A.10) indicate that *F. oxysporum* VOCs affect many genes/processes in *Trichoderma*, not just those associated with the production of antifungal molecules and biocontrol. This is in agreement with other reports showing that a diverse array of genes involved in metabolism, transcription, cellular transport, and signal transduction were differentially regulated during mycoparasitic interactions (Atanasova et al., 2013; Morán-Diez et al., 2019; Seidl et al., 2009). However, other than the treatment of 100 μmol/L 4-ethylanisole, which appeared to induce stress response in *T. harzianum* (Fig. 5 and Fig. A.5) as previously reported (Atanasova et al., 2013; Seidl et al., 2009), we did not find strong evidence suggesting that VOCs trigger stress response in *Trichoderma.* As culture-derived VOCs contain multiple potentially bioactive VOCs, we anticipated that culture-derived VOCs would affect more genes than individual compounds, which was the pattern observed (Fig. 4**C**). Since the composition and abundance of VOCs vary among different *F. oxysporum* strains (Li et al., 2018) and different compounds likely affect different sets of genes in individual species, many of the DEGs and enriched GO terms are unique to each VOC treatment (Fig. 5 and Figs. A.3-A.6).

Although the DEGs and enriched GO terms were not congruent with the degree of antifungal molecule secretion observed, some of them were significantly affected by multiple VOC treatments, including proteolysis process (in *T. virens* and *T. viride*), organonitrogen compound biosynthetic process (in *T. virens* and *T. viride*), and organic acid metabolic process (in *T. asperellum*) (Fig. 5**)**. Various groups of protease/peptidase genes were up-regulated in the four *Trichoderma* spp. following specific VOC treatments (Figs. A.7-A.10), which is in agreement with previous studies that indicated an important role of proteases in the early stage of mycoparatism in *Trichoderma* spp. (Morán-Diez et al., 2019; Seidl et al., 2009; Vieira et al., 2013). The expression of genes encoding cell wall degrading enzymes (e.g. chitinases, β-glucanase, and chitosanase) was shown to be differentially regulated before *Trichoderma* spp. in contract with different pathogens (Li et al., 2018; Morán-Diez et al., 2019; Vieira et al., 2013). Consistent with previous results, we saw both up- and down-regulation of genes encoding cell wall degrading enzymes in the four *Trichoderma* spp. when exposed to different VOCs (Table 1).

Analysis of genes associated with secondary metabolism (Figs. 6 and 7) revealed differential expression of genes encoding NRPS, PKS, and terpene cyclase in the four *Trichoderma* spp. (Fig. 6). Non-ribosomal peptides, polyketides, and terpenes represent the main antibiotic metabolites produced by *Trichoderma* spp. and are produced by secondary metabolite biosynthetic genes located on gene clusters (Mukherjee et al., 2012). However, functional characterization of secondary metabolite gene clusters in *Trichoderma* is inadequate, thus making it challenging to predict what antifungal molecules are differentially produced in response to VOCs. A terpene gene cluster in *T. virens* was induced by 3-methyl-1-butanol and isopentyl acetate but suppressed or not affected by other treatments (Fig. 7**B**). This gene cluster was suggested to be involved in the production of two different categories of terpene compounds, monoterpenes and sesquiterpenes, in *T. virens* strain Gv29-8 (Crutcher et al., 2013). However, the role of individual terpene compounds in biocontrol remains to be determined. More studies are needed to understand why the expression of these gene clusters exhibits such patterns of response and whether any of the differentially expressed gene clusters are involved in biocontrol.

The multifaceted effects of *F. oxysporum* VOCs, individually and collectively, on *Trichoderma* spp. suggest that some VOCs likely affect the biocontrol efficacy. However, validation of their significance in biocontrol and elucidation of their mechanisms of action require further investigation. Because our experiments were based on fungal strains cultured on PDA (Fig. 1), we do not know whether *F. oxysporum* produces candidate bioactive VOCs at levels high enough to affect *Trichoderma* BCAs in soils and whether edaphic factors influence the production of such VOCs and *Trichoderma* responses to specific VOCs. Several tools (Kellogg and Kang, 2020; Tholl et al., 2021) are available for analyzing soil VOCs. Analysis of VOCs under diverse conditions (e.g., different soils, fertilization, pH, presence/absence of plants), combined with assessing plant growth/health and fungal biomass, can help understand how various environmental factors affect VOC production/response. The application of specific VOCs to soils containing *Trichoderma* and the subsequent quantification of their biomass can help evaluate their role in antibiosis. We designed a simple system that can help perform this evaluation (Li and Kang, 2022). The importance and effects of specific VOCs can also be evaluated by disrupting or increasing their production via targeted manipulation of the genes involved in their production.

Soil microbiomes play vital roles in plant growth and health (Trivedi et al., 2020) and are likely to influence biocontrol efficacy in multiple ways. Considering that some VOCs affect diverse fungi and bacteria (Kang et al., 2021; Li et al., 2019), another crucial question is whether VOCs released by *F. oxysporum* and *Trichoderma* affect plant health and biocontrol by modifying the composition and activity of soil and rhizosphere microbiomes. Multiple tools (Kang et al., 2022; Kellogg and Kang, 2020; Trivedi et al., 2020) are available to help analyze the response of microbes to VOCs. Since *F. oxysporum* and *Trichoderma* can influence neighboring microbes via other mechanisms (e.g., secretion of water-borne antimicrobial metabolites and enzymes and physical competition), an experimental setup that allows measurement of only VOC-mediated effects is needed. The system described by Li and Kang (2022) meets this need, but lacks the capability to determine the concentration and temporal change of applied VOCs in treated soils. Some of the tools described by Tholl et al. (2021) can be modified to meet both needs.

Although the available information on how VOCs modulate cellular processes is very limited, we can infer their potential mechanisms from the available knowledge on how metabolites regulate cell signaling and gene expression, which have been shown to bind to protein sensors or regulatory proteins, function as enzyme cofactors, and chemically modify proteins (Figlia et al., 2020; Wang and Lei, 2018). The responses of *T. virens* to 4-ethylanisole and 3-methyl-1-butanol were not distinct from those of their structural isomers (Fig. A.2), suggesting that compound-specific receptors are unlikely to be involved. Owing to their physicochemical properties, VOCs can enter microbial cells via passive diffusion through the plasma membrane (Weisskopf et al., 2021) and interact with various cellular components. Because we only used one time point for VOC treatment, we cannot differentiate initial changes in gene expression from those triggered by the initial changes. Identification of the DEGs at multiple time points will help understand the sequence of molecular changes caused by individual compounds, why some compounds cause opposite effects on antifungal molecule secretion depending on the species and VOC concentration, and why different species distinctly respond to each compound.

## CRediT authorship contribution statement

**Ningxiao Li:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Formal analysis, Data curation. **Ki-Tae Kim:** Writing – review & editing, Software, Resources, Formal analysis. **Carl Schlagnhaufer:** Validation, Investigation. **Seogchan Kang:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biocontrol.2024.105473.

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