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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Transcriptome analysis of *Botrytis cinerea* in response to tea tree oil and its two characteristic components

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Abstract

Tea tree oil (TTO) and its two characteristic components (terpinen-4-ol and 1,8-cineole) have been shown to inhibit *Botrytis cinerea* growth. In this study, we conducted a transcriptome analysis to determine the effects of TTO and its characteristic components, alone and in combination, against *B. cinerea*. Most differentially expressed genes (DEGs) from *B. cinerea* cells treated with terpinen-4-ol participated in the biosynthesis of secondary metabolites, and the metabolism of amino acids, carbohydrates, and lipids. All treatments containing terpinen-4-ol potentially induced mitochondrial dysfunction and oxidative stress. These were further confirmed by the decreased activities of several enzymes (e.g., succinate dehydrogenase (SDH), malate dehydrogenase (MDH), α-ketoglutarate dehydrogenase (α-KGDH), isocitrate dehydrogenase (ICDH)), the increased activities of certain enzymes (e.g., catalase (CAT), peroxidase (POD), superoxide dismutase (SOD)), and increased content of hydrogen peroxide (H_2O_2). 1,8-Cineole mainly affected DEGs involved in genetic information processing, resulting in cell death. This study provides insight into the molecular mechanism of *B. cinerea* inhibition by TTO, and explains the synergistic effect of terpinen-4-ol and 1,8-cineole on *B. cinerea*.

Keywords Transcriptomics · Tea tree oil · *Botrytis cinerea* · 1,8-Cineole · Terpinen-4-ol

Introduction

Fungal infection is one of the major causes of postharvest decay in fruit. Postharvest growth of *Botrytis cinerea*, a common fungus that is responsible for gray mold disease, results in considerable damage to a wide range of fruits and vegetables (Saito et al. 2016). Chemical fungicides are primarily used to control this pathogen (Rosslenbroich and Stuebler 2000). However, growing concerns of using such fungicides are including risks to human health, contamination of the environment, chemical residues, and the increase of fungicide-resistant strains. These prompt researchers to develop new antimicrobial agents (Cabral et al. 2013). Essential oils have received much attention in the food industry due to their promising antifungal, eco-friendly, and biodegradable properties (Tzortzakis and Economakis 2007). One well-known example is tea tree oil (TTO), also known as *Melaleuca alternifolia* oil, which is extracted via steam distillation from the leaves of tea trees grown in Australia. Gas chromatography-mass spectrometer (GC-MS) analysis suggests that TTO contains more than 100 compounds, including approximately 50% monoterpene hydrocarbons, ~50% monoterpene hydrocarbon oxygenated derivatives, and a small amount of sesquiterpenes (Swords and Hunter 1978). The international standard regulation for TTO sets a minimum content of 30% terpinen-4-ol and a maximum content of 15% 1,8-cineole (Hammer et al. 2006). TTO has both antiviral and anti-inflammatory bioactivities; however, it has recently attracted increasing attention for its antifungal activity (de Groot and Schmidt 2016; Hammer et al. 2006; Sharifi-Rad et al. 2017). The previous studies have found that TTO controls the growth of *B. cinerea* more effectively than other essential oils (e.g., pine, cinnamon, clove, garlic oil) (Cheng and Shao 2011; Szczerbanik et al. 2007). TTO can also effectively control gray mold disease in strawberry and cherry fruit (Li et al. 2017a; Shao et al. 2013b).

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Essential oils and their constituents are thought to disrupt microbial cell walls and membranes, causing the loss of intracellular substances and ultimately cell death (Hyldgaard et al. 2012; Souza et al. 2016; Sun et al. 2015; Xing et al. 2014). Plant essential oils and their components also exert antifungal activity by influencing mitochondrial morphology and function (Jun et al. 2012; Tao et al. 2014; Zheng et al. 2015). Our previous studies confirmed that TTO primarily targets the *B. cinerea* cell wall and then changes membrane fatty acid composition, which increases membrane permeability, leading to the fatal release of cellular material (Shao et al. 2013a). TTO also causes mitochondrial damage in *B. cinerea*, disrupting the tricarboxylic acid (TCA) cycle and increasing levels of reactive oxygen species (ROS) (Li et al. 2017b). Proteomics and metabolomics analyses have also shown that TTO can disrupt the TCA cycle and cause mitochondrial dysfunction in *B. cinerea* (Xu et al. 2017a, 2017b). Terpinen-4-ol, the major characteristic component in TTO, exhibits the highest antifungal activity and acts mainly on the cell membranes of *B. cinerea*. The other characteristic component, 1,8-cineole, damages cellular organelles and acts synergistically with terpinen-4-ol (Yu et al. 2015). However, the mechanisms of TTO and its components affecting fungal cells at the molecular level have not been well determined.

In order to further study antifungal mechanisms, high-throughput RNA sequencing has been used to examine mRNA expression profiles in fungal cells treated with essential oils. Ouyang et al. (2016) used transcriptional profiling to demonstrate that citral inhibits *Penicillium digitatum* by downregulating ergosterol biosynthesis genes. Zhou et al. (2018) demonstrated that decanal downregulates genes associated with oxidative phosphorylation, transcription, and translation in *Penicillium expansum*, and is partly responsible for the growth inhibition observed after treatment with this essential oil. In cinnamaldehyde and citral-treated *P. expansum*, genes that affects amino and nucleotide sugar metabolism, ergosterol biosynthesis, energy and amino acids metabolism, and ATP-binding cassette transporters was also found to be downregulated (Wang et al. 2018).

RNA-Seq has not yet been used to examine the mechanisms by which TTO and its two characteristic components (i.e., terpinen-4-ol and 1,8-cineole) impair *B. cinerea* growth and viability. This study therefore used RNA-seq to determine the effects of TTO and its two characteristic components, alone and in combination, on the growth, oxidative damage, and TCA cycle of *B. cinerea*. The whole gene expression profile provides information about the molecular mechanism of TTO against *B. cinerea*, and reveals the synergistic effect of the two characteristic components.

Materials and methods

Pathogen and chemicals

B. cinerea (ACCC 36028) was purchased from the Agricultural Culture Collection of China, Beijing, China and cultured at 25 °C on potato dextrose agar medium (PDA; containing 1 L of an infusion from potatoes, 20 g/L glucose, and 15 g/L agar) before use in experiments. Spores were harvested by flooding a plate with 10 mL 0.9% sterile NaCl solution. The spore suspension was adjusted to 5×10^6 spores/mL using a hemocytometer. One milliliter of the suspension was inoculated into 250 mL flasks containing 150 mL sterile potato dextrose broth (PDB: containing 1 L of an infusion from potatoes, 20 g/L glucose) medium and cultured at 25 °C on a rotary shaker at 150 rpm (rpm) for 3 days before mycelia were harvested. TTO was purchased from Fuzhou Merlot Lotus Biological Technology Company, Fujian, China. The TTO contained 37.11% terpinen-4-ol and 4.97% 1,8-cineole, as specified by the supplier. Terpinen-4-ol (95%) and 1,8-cineole (98%) were purchased from TCI Shanghai, Shanghai, China. Pyrimethanil (as Scala, 40%, active ingredient) was obtained from Bayer Crop Science China, Hangzhou, China.

Antifungal effects of TTO and its two characteristic components on *B. cinerea*

The effect of TTO and its two characteristic components on mycelial growth of *B. cinerea* was determined using the agar dilution method as described by Yu et al. (2015). TTO, 1,8-cineole, terpinen-4-ol, a 1:1 (*v*/v) mixture of 1,8-cineole, and terpinen-4-ol, or pyrimethanil were mixed with PDA medium with 1% Tween-80 to obtain concentrations of 0.00, 0.25, 0.50, and 1.00 μL/mL. The medium was then poured into sterilized Petri dishes. Pyrimethanil, a commercial fungicide, was applied as a positive control. PDA medium without TTO or characteristic component was used as a control. A 5-mm-diameter disc containing *B. cinerea* was cut from the edge of a growing fungal culture and placed at the center of each plate. The plates were then incubated at 25 °C for 3 days and each treatment was performed in quintuplicate. The percentage of mycelial growth inhibition (MGI) was calculated according to the formula:

$$\mathrm{MGI}\ (\%) = [(\mathrm{dc}-\mathrm{dt})/\mathrm{dc}] \times 100$$

where dc (cm) is the mean colony diameter on the control plates and dt (cm) is the mean colony diameter on plates receiving treatment.

Transcriptome analysis

Sample preparation

A *B. cinerea* culture in PDB was incubated 3 days. Mycelia were harvested by centrifuging the *B. cinerea* culture at 8000×*g* for 10 min and rinsing three times with 0.1 M phosphate-buffered saline (PBS) (pH 7.4). Equal amounts (wet weight) of mycelia were resuspended in the same volume of PDB medium containing TTO, 1,8-cineole, terpinen-4-ol, or combined 1,8-cineole and terpinen-4-ol (final concentration 0.5 μL/mL). Samples resuspended in PDB medium containing no TTO or components were used as controls. Mixtures were then incubated for 2 h at 25 °C with shaking at 150 rpm. Mycelia were harvested and rinsed three times with 0.1 M PBS (pH 7.4). All collected mycelia were stored at − 80 °C for RNA extraction. Three technical replicates were performed for each sample, and each treatment was performed in triplicate.

Total RNA isolation, library construction, and sequencing

Total RNA was extracted from control mycelia, and mycelia treated with 0.5 mL/L TTO, 1,8-cineole, terpinen-4-ol, or combined 1,8-cineole and terpinen-4-ol. The extraction protocol followed the instructions accompanying the Trizol Reagent (Life Technologies, Carlsbad, USA). RNA integrity and concentration were checked using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, USA). mRNA was isolated using a NEBNext Poly (A) mRNA Magnetic Isolation Module (NEB, E7490). The cDNA library was constructed using a NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, E7530) and NEBNext Multiplex Oligos for Illumina (NEB, E7500), following the manufacturer's instructions (New England Biolabs, Inc., Ipswich, USA). Briefly, the enriched mRNA was fragmented into approximately 200 nt lengths. The fragments were used for first-strand cDNA synthesis, and then second strand cDNA synthesis was performed. The double-stranded cDNA was subjected to reactions to perform end-repair, dA-tailing, and adaptor ligation. The modified fragments were isolated using Agencourt AMPure XP beads (Beckman Coulter, Inc., Brea, USA), and enriched by PCR amplification. Finally, the cDNA libraries were sequenced on a flow cell using an Illumina HiSeq™ 2500 sequencing platform (Biomarker Tech, Beijing, China). For each treatment, three samples were prepared in parallel for transcriptomics analysis.

Transcriptomic analysis using reference genome-based reads mapping

Raw reads containing adaptor sequences, unknown nucleotides (> 5% of the read), or reads in which more than 20% of the nucleotides had low Q-value scores (≤ 20) were removed using a Perl script. The clean reads were mapped to the *B. cinerea* genome (https://www.ncbi.nlm.nih.gov/genome/?term=Botrytis+cinerea) using Tophat2 (Kim et al. 2013). The aligned records from the aligners in BAM/SAM format were further examined to remove potential duplicate molecules. Gene expression levels were estimated using FPKM values (fragments per kilobase of exon per million fragments mapped) using Cufflinks (Trapnell et al. 2010). The RNA-Seq data have been deposited in the NCBI Gene Expression Omnibus with the accession number GSE138291.

Identification of differentially expressed genes

DESeq was used to evaluate differential gene expression between the control and treated samples. Differences were calculated based on the ratio of the Fragments Per kb per Million reads (FPKM) values. The false discovery rate (FDR) control method was used to identify the threshold *p* value in multiple tests in order to compute the significance of the differences. The classification criteria for DEGs were FDR ≤ 0.001 and absolute value of $|\log_2(\text{fold change})| \geq 1$.

Sequence annotation

Genes were compared against several protein databases by BLASTX, including the National Center for Biotechnology Information (NCBI) non-redundant protein (Nr) database and the Swiss-Prot database. Alignments were required to have *E* values less than or equal to 10^{-5}. Nucleotide sequences for genes were also used as queries against the NCBI non-redundant nucleotide sequence (Nt) database using BLAST (*E* value less than or equal to 10^{-5}). Genes were tentatively identified based on the alignment with the highest BLAST score and the accompanying sequence annotation for the match.

To associate the gene with gene ontology (GO) terms, the Nr BLAST results were imported into the Blast2GO program (Conesa et al. 2005). This analysis maps all the annotated genes to GO terms in the database and counts the number of genes associated with each term. A Perl script was then used to plot GO functional classification for the unigenes with a GO term hit to view the distribution of gene functions. The preliminary annotation was enriched and refined using TopGo (R package). Gene sequences were also aligned to the Clusters of Orthologous Group (COG) database to predict and classify functions (Tatusov et al. 2000). GO enrichment analysis was implemented using the GOseq R packages, based on the

Wallenius non-central hyper-geometric distribution (Young et al. 2010). KOBAS was used to test the statistical significance of DEG enrichment in KEGG pathways (Xie et al. 2011).

Quantitative real-time reverse transcription PCR analysis

Total RNA was extracted from each sample using Trizol regent (Invitrogen, Carlsbad, USA) following the manufacturer's instructions. RNA was quantified with NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA) and checked for integrity by agarose gel electrophoresis. cDNAs were synthesized using SuperMix (Vazyme, Nanjing, China) following the manufacturer's instructions. All cDNA was diluted 30-fold prior to use. The quantitative real-time reverse transcription PCR (qRT-PCR) reaction mixture (20 μL total volume) contained 10 μL Master Mix (Vazyme, Nanjing, China), 0.8 μL each primer (10 μM), 0.4 μL 50 × ROX Reference Dye 2 (Vazyme, Nanjing, China), 5 μL cDNA, and 3.6 μL ddH_2O. The qRT-PCR reaction conditions were as follows: 95 °C for 30 s, 40 cycles of 95 °C for 10 s, and 60 °C for 30 s. Gene expression was normalized to the reference gene ubiquitin (UBQ) and calculated using the $2^{-\Delta\Delta Ct}$ method (Ren et al. 2017). All qRT-PCR reactions were carried out in triplicate. Primers are listed in Supplementary Table S1.

Activity of key enzymes involved in the TCA cycle

As described above, mycelia were treated with TTO, 1,8-cineole, terpinen-4-ol, or combined 1,8-cineole and terpinen-4-ol in PDB medium at a final concentration of 0.50 μL/mL. After treatment, mycelia were harvested and washed with 0.1 M PBS (pH = 7.4) three times and then ground in liquid nitrogen. The ground material was suspended in PBS (pH = 7.4) and centrifuged at 10,000×g for 10 min at 4 °C. Enzyme activities were measured in the supernatant for MDH, SDH, ICDH, and α-KGDH, using commercially available kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Assay protocols followed the manufacturer's instructions. MDH, ICDH, and α-KGDH activities were detected at 340 nm in redox reaction assays. SDH activity was determined at 600 nm. Enzyme activity was expressed as units per milligrams of protein (U/mg prot). All tests were performed in triplicate.

Determination of hydrogen peroxide accumulation and assay of catalase, peroxidase, and superoxide dismutase activities

As described above, mycelia were treated with TTO, 1,8-cineole, terpinen-4-ol, or combined 1,8-cineole and terpinen-4-ol. After treatment, the mycelia (1 g wet weight) were mixed with 5 mL 0.05 M PBS (pH = 7.0, containing 3% polyvinyl pyrrolidone), subjected to ultrasonic grinding for 15 min, and centrifuged at 10,000×g for 10 min at 4 °C. The supernatant was used to determine hydrogen peroxide (H_2O_2) content and enzyme activity. H_2O_2 content and superoxide dismutase (SOD) activity was determined using assay kits obtained from Nanjing Jiancheng Institute of Bioengineering (Nanjing, Jiangsu, China), following the manufacturer's instructions. H_2O_2 content was expressed as mmol/g protein. SOD activity was determined at 560 nm and was defined as U/mg protein.

Catalase (CAT) activity was determined by adding 0.2 mL of the enzyme preparation to 2.8 mL of reaction mixture containing 2.6 mL 0.05 M PBS and 0.2 mL of substrate 0.75% (*v*/*v*) H_2O_2 solution. One unit of CAT activity was defined as the amount of enzyme required to change absorbance at 240 nm at a rate of 0.01 per min.

Peroxidase (POD) activity was determined by adding 0.1 mL of the crude enzyme extract to 1.9 mL of reaction mixture containing 0.8 mL 0.02 M sodium phosphate buffer, 1 mL 0.25% (*v*/*v*) guaiacol, and 0.1 mL 0.75% (*v*/*v*) H_2O_2. One unit of POD activity was defined as the amount of enzyme required to increase absorbance at 470 nm by 0.001 per min.

Statistical analysis

All experiments were carried out in triplicate. Mean values and standard deviations were calculated using Excel 2010 (Microsoft Inc., Seattle, WA, USA). Statistical analysis was performed using SAS (Version 8.2; SAS Institute, Cary, NC, USA). Data were analyzed by one-way analysis of variance (ANOVA). Comparison of means was performed by Duncan's multiple range tests. A value of $p < 0.05$ was considered statistically significant.

Results

Antifungal activity of TTO and its two characteristic components on *B. cinerea*

TTO and its two characteristic components (i.e., 1,8-cineole, terpinen-4-ol) were tested for their ability to inhibit *B. cinerea* growth using a plate growth assay. Mycelial growth of *B. cinerea* was reduced by TTO and its two characteristic components in a dose-dependent manner (Table 1). For each concentration, compared with other treatments, 1,8-cineole exhibited lowest antifungal activity. However, the antifungal activity of 1,8-cineole dramatically increased when combined with terpinen-4-ol, and was significantly higher than single component. For example, the inhibitory effect of 1,8-cineole at 0.25 μL/mL increased from 13.43 to 54.98%, which is clearly

Table 1 Antifungal activity of TTO and its two characteristic components and pyrimethanil against *B. cinerea*

Concentration (μL/mL)	Mycelial growth inhibition (%)*				
	TTO	1,8-Cineole	Terpinen-4-ol	1,8-Cineole + terpinen-4-ol	Pyrimethanil
0.00	0.00 ± 0.10d	0.00 ± 0.20d	0.00 ± 0.10d	0.00 ± 0.10c	0.00 ± 0.10c
0.25	13.43 ± 4.08c	5.90 ± 5.65c	32.20 ± 2.40c	54.98 ± 3.01b	85.61 ± 3.78b
0.50	25.73 ± 3.02b	18.82 ± 2.13b	78.87 ± 0.64b	100.00 ± 0.00a	96.31 ± 0.33a
1.00	59.77 ± 5.50a	29.75 ± 5.44a	100.00 ± 0.00a	100.00 ± 0.00a	100.00 ± 0.00a

*Values are presented as mean ± SD

a–d Significant differences at $p < 0.05$ level

higher than that of terpinen-4-ol alone. At 0.50 μL/mL, combined 1,8-cineole and terpinen-4-ol completely inhibited the growth of mycelial and its antifungal activity was comparable to that of pyrimethanil. The results demonstrated that terpinen-4-ol acts synergistically with 1,8-cineole and these two characteristic components in combination were more effective than TTO and other commercial fungicides.

Sequencing data

After high-throughput RNA sequencing, reads were filtered using the criteria described in Materials and Methods, yielding 111.22 Gb of clean sequence data. The GC content for all samples exceeded 45%. The Q20 ratio (used to evaluate reads quality) was greater than 94%, and Q30 base percentage was at least 87.07%. Bulk sequencing statistics are summarized in Supplementary Table S2. The sequencing data are of good quality and are suitable for the analyses described below.

Differentially expressed genes

Differentially expressed genes were identified by comparing RNA sequence data from *B. cinerea* treated with TTO, 1,8-cineole, terpinen-4-ol, or combined 1,8-cineole and terpinen-4-ol. After applying the classification criteria ($p \leq 0.05$ and $|\log_2$ (fold change) $| \geq 1$), 855 DEGs were identified (439 up- and 416 downregulated) between the untreated (control) and the four treated samples (Fig. 1a). The Venn diagram in Fig. 1b highlights the unique and shared genes found in each comparison. The control vs. TTO yielded the highest number of unique DEGs (164), followed by control vs. combined 1,8-cineole and terpinen-4-ol (158), control vs. terpinen-4-ol (90), and control vs. 1,8-cineole (49). The highest number of shared genes were identified between control vs. TTO and control vs. terpinen-4-ol (23), followed by control vs. TTO and control vs. 1,8-cineole (15). Only 5 DEGs were shared by all comparisons, but all are predicted/hypothetical proteins. Figure 1c summarizes the numbers of up- and downregulated DEGs in the four treatment samples vs. the control. In the control vs. TTO comparison, 280 DEGs were identified (105 upregulated and 175 downregulated). The smallest numbers of DEGs (94) were found in the control vs. 1,8-cineole sample (36 upregulated and 58 downregulated). When the control was compared with samples treated with terpinen-4-ol, 201 DEGs were found (125 upregulated and 76 downregulated). Finally, in the comparison between the control vs. the combined 1,8-cineole and terpinen-4-ol, 280 DEGs were identified, of which 173 were upregulated and 107 were downregulated. Supplementary Table S3 lists all DEGs of *B. cinerea* after treatment with TTO, 1,8-cineole, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol.

Functional annotation of DEGs

Based on GO functional analysis, DEGs were classified into three major categories: molecular function, cellular component, and biological process (Fig. 2). The analysis classified 110 DEGs from the control vs. TTO sample, 38 from control vs. 1,8-cineole, 82 from control vs. terpinen-4-ol, and 116 from control vs. combined 1,8-cineole and terpinen-4-ol. In the molecular function category, catalytic activity was the most frequent term, followed by binding and transporter activity. In the cellular component category, the most highly enriched terms were membrane and membrane part in control vs. TTO, control vs. terpinen-4-ol, and control vs. combined 1,8-cineole and terpinen-4-ol. However, for the control vs. 1,8-cineole sample, the cell part, cell, and organelle were the most highly enriched terms. In the biological process category, the metabolic processes, single-organism processes, and cellular processes were the most highly enriched in all comparisons.

The DEGs were also examined in a KEGG pathway analysis (Fig. 3). Forty-one DEGs were annotated to 39 KEGG pathways involved in metabolism, cellular processes, and genetic information processing. In the DEGs from the control vs. TTO sample, the most dominant pathways were annotated as fatty acid metabolism, pyruvate metabolism, and peroxisome. In the control vs. 1,8-cineole sample, few DEGs mapped to a pathway, but the most enriched pathways were identified as glycine, serine, and threonine metabolism, and nucleotide

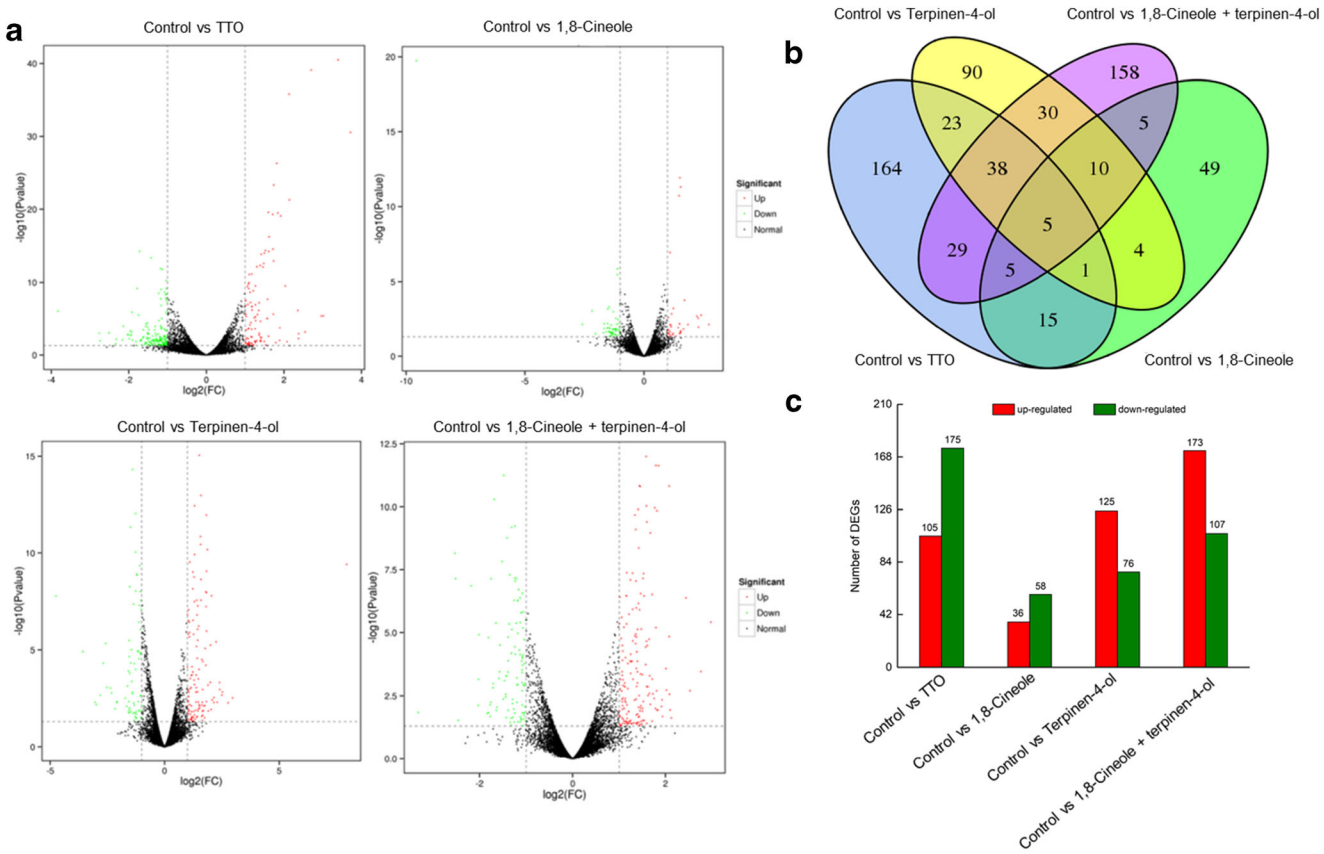

Fig. 1 DEGs detected in comparisons of untreated control vs. treated samples. **a** Volcano plots for DEGs. *B. cinerea* was treated with TTO, 1,8-cineole, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol. Horizontal and vertical dotted lines represent DEG classification thresholds for fold-difference and *p* value. Red and green dots represent genes with increased or decreased expression (treated/control), respectively. **b** Venn diagram showing unique and shared DEGs. **c** Numbers of DEGs identified in each of the four comparisons. Color-coding is identical to that used in panel **a**

excision repair. In control vs. terpinen-4-ol, the most dominant pathways included fatty acid metabolism, fatty acid degradation, glycosylphosphatidy linositol (GPI)–anchor biosynthesis, and pyruvate metabolism. Finally, in the control vs. combined 1,8-cineole and terpinen-4-ol, the most enriched pathways were involved in fatty acid degradation, pyruvate metabolism, peroxisome, and GPI–anchor biosynthesis.

Analysis of DGEs

Genes related to the cell wall

Expression of several genes involved in integrity and biogenesis of the cell wall were affected by treatment with TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol (Fig. 4a). Genes involved in the regulation of cell wall integrity (e.g., *PIL1*, BC1G_01023; *LSP1*, BC1G_15020; *RBE1*, BC1G_08280; *MKC1*, BC1G_15855; *gim-1*, BC1G_00054) were generally upregulated in *B. cinerea* cells treated with terpinen-4-ol and combined 1,8-cineole and terpinen-4-ol. In TTO-treated cells, only *PIL1* (BC1G_01023) was upregulated (2.13-fold). Genes involved in cell wall biogenesis/degradation were downregulated in TTO-treated cells. These include *EXG1* (BC1G_02731), *agn1* (BC1G_07058), and *RHO2* (BC1G_0182). Transcription factors (*ACE2*, BC1G_12819) that regulate expression of genes involved in cell separation and other cell wall related genes were downregulated in cells treated with TTO and combined 1,8-cineole and terpinen-4-ol. Overall, in addition to the 1,8-cineole, the oil containing terpinen-4-ol all affected the expression of the cell wall-related genes.

Genes related to the cell membrane

Gene *FAS2* (BC1G_14055) and the delta (12) fatty acid desaturase gene (BC1G_14126), both involved in fatty acid synthesis, were downregulated in *B. cinerea* cells treated with TTO and terpinen-4-ol, respectively (Fig. 4b). The gene *CYP505* (BC1G_11409), related to fatty acid degradation, was downregulated 2.29-fold and 2.35-fold in terpinen-4-ol and combined 1,8-cineole and terpinen-4-ol-treated *B. cinerea* cells. The gene *ayr1* (BC1G_10896), involved in the

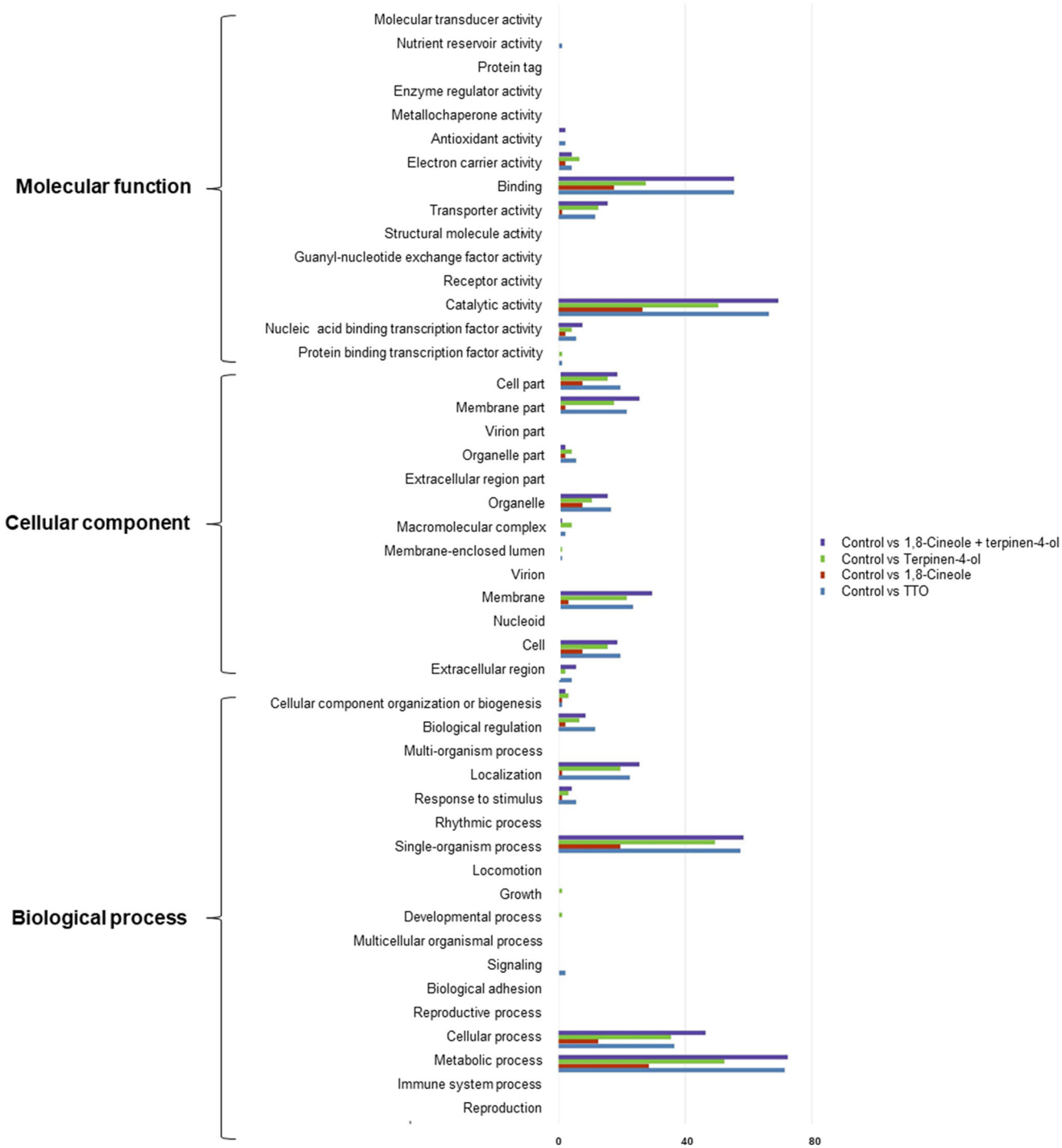

Fig. 2 GO classification analysis of DEGs in the four treatment groups vs. untreated control. Treatments are represented using different colors

biosynthesis of phosphatidic acid, was upregulated 2.63-fold in combined 1,8-cineole and terpinen-4-ol-treated *B. cinerea* cells. Gene *UPC2* and *ATG26* (BC1G_01004), associated with regulation of sterol biosynthesis, were upregulated in *B. cinerea* cells after exposure to TTO and combined 1,8-cineole and terpinen-4-ol, respectively. However, no obvious change in expression for any of these six genes was observed in cells treated with 1,8-cineole.

Genes related to mitochondrial function

Expression of several genes involved in mitochondrial function changed after treatment with TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol (Fig. 4c). Genes encoding NAD-dependent malic enzyme (*mae2*, BC1G_13155), intron-encoded endonuclease (*bI1*, SS1G_06351), and NADH-ubiquinone oxidoreductase

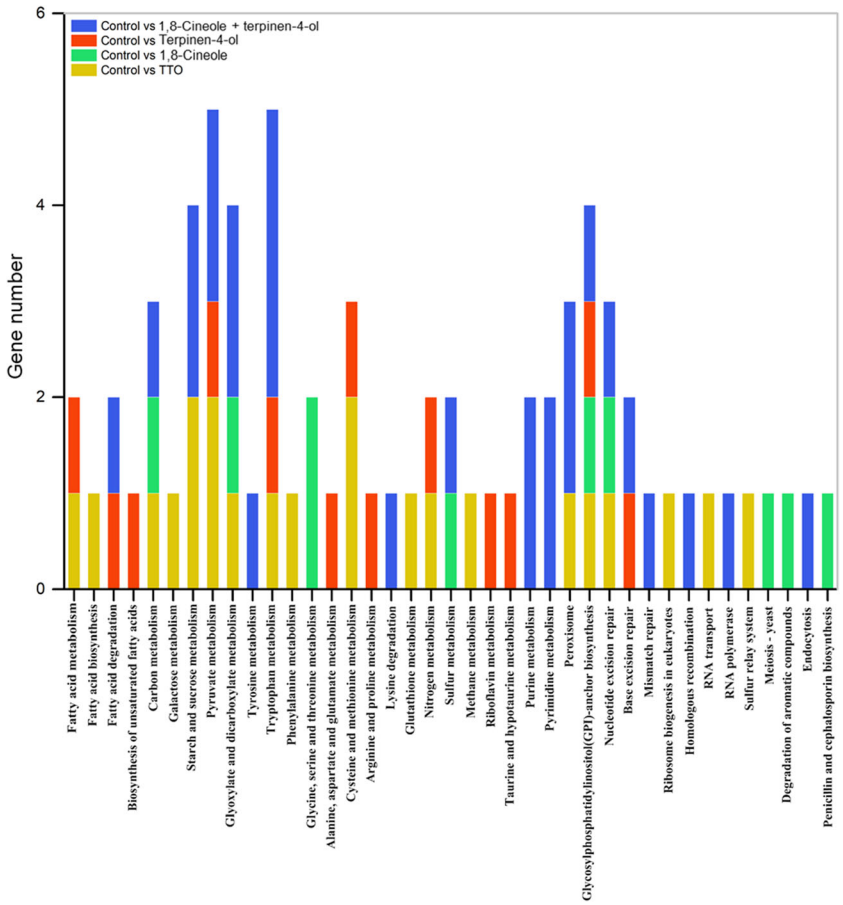

Fig. 3 KEGG pathway analysis showing numbers of DEGs (*y*-axis) and pathway classifiers (*x*-axis) enriched from four treatment groups. Treatments are represented by different colors

chain 2 (*ND2*) were downregulated 2.10-fold, 2.61-fold, and 2.79-fold in *B. cinerea* cells treated with TTO, respectively. These three genes (*mae2*, *bI1*, and *ND2*) were also downregulated 3.50-fold, 5.81-fold, and 5.75-fold after exposure to combined 1,8-cineole and terpinen-4-ol, respectively. With terpinen-4-ol treatment, a mitochondrial translational initiation gene (*mrp51*, BC1G_03442), a gene encoding a transmembrane chaperone for the mitochondrial inner membrane (*BCS1*, BC1G_09262), and a nuclear-encoded mitochondrial enzyme gene (*prnC*, BC1G_12887) were downregulated 5.92-fold, 2.90-fold and 2.04-fold, respectively. *mmf1*, which plays a role in the maintenance of mitochondrial DNA, was upregulated 2.55-fold, 2.17-fold, and 2.59-fold in *B. cinerea* cells treated with TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol, respectively. The gene *coq3* (BC1G_08822), which encodes a protein involved in the ubiquinone biosynthesis pathway, was upregulated 2.10-fold in response to TTO. *hmt2* (BC1G_10152), which encodes a mitochondrial enzyme that is a sulfide-quinone oxidoreductase, was upregulated 2.05-fold in cells treated with 1,8-cineole. However, this treatment did not affect the other genes involved in mitochondrial function.

Genes related to the general stress response

The relative expression of stress-related genes is shown in Fig. 4d. One gene (*ish1*, BC1G_03275) encoding a stress response protein was upregulated 4.08-fold, 4.13-fold, and 3.12-fold in cells treated with TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol, respectively. Under TTO treatment, genes encoding putative peroxiredoxin (*AN8692*, BC1G_09932), glutathione reductase (*gtr-1*, BC1G_15101), and catalase (*cta1*, BC1G_09386) were upregulated 3.06-fold, 4.39-fold, and 2.14-fold, respectively. In contrast, the gene encoding flavohemoprotein (*yhb1*, BC1G_03891) was downregulated 2.65-fold. Upregulation of *yhb1* and *cta1* were observed in *B. cinerea* cells treated with combined 1,8-cineole and terpinen-4-ol. However, no detectable changes in expression for these stress-related genes were observed in cells treated with 1,8-cineole.

Genes related to genetic information processing

Essential oil stress appears to affect genes related to genetic information processing (transcription, translation, replication, and repair) (Fig. 4e). One gene *uaY* (BC1G_02267), involved in positive regulation of

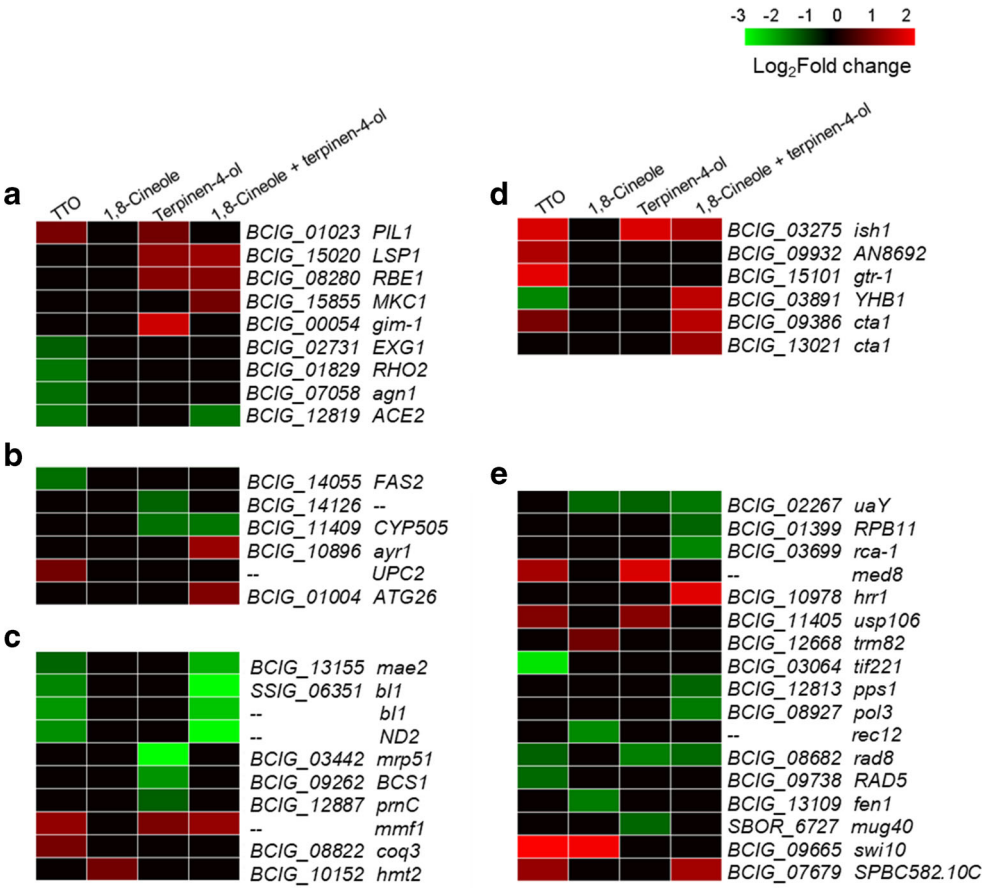

Fig. 4 Heatmaps showing relative expression for selected DEGs. The Log_2Fold change was colored. Colors indicate differential gene expression (RNA-Seq) in treated *B. cinerea* cells vs. non-treated (control) cells. Green: downregulated; red: upregulated; black: not differentially expressed. Each horizontal row represents one DEG, and vertical columns represent treatments with TTO, 1,8-cineole, terpinen-4-ol, or combined 1,8-cineole and terpinen-4-ol. Shown are DEGs involved in **a** cell wall, **b** cell membrane, **c** mitochondrial function, **d** stress response, and **e** genetic information processing

transcription, was downregulated in cells treated with 1,8-cineole, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol. Gene *med8* (BofuT4P101000020001), a co-activator involved in the transcription of nearly all RNA polymerase II-dependent genes, was upregulated in cells treated with TTO and terpinen-4-ol. Gene *usp106* (BC1G_11405), involved in mRNA processing, was up-regulated 2.25-fold and 2.34-fold under TTO and terpinen-4-ol stress, respectively. Among genes related to translation, *trm82* (BC1G_12668), encoding tRNA modification protein, was upregulated 2.02-fold under 1,8-cineole stress, while translation initiation factor eIF-2B (*tif221*, BC1G_03064) was downregulated 4.97-fold in response to TTO treatment. Expression of genes involved in DNA synthesis (*PPS1*, BC1G_12813; *pol3*, BC1G_08927) was downregulated in cells treated with combined 1,8-cineole and terpinen-4-ol. The gene *rec12* (BofuT4_P107440.1), involved primarily in the early steps of meiotic recombination, was downregulated 2.71-fold under 1,8-cineole stress. Genes involved in DNA repair, such as *rad8* (BC1G_08682), *RAD5* (BC1G_09738), *fen1* (BC1G_13109), and *mug40* (SBOR_6727), were downregulated in cells treated with TTO, 1,8-cineole, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol. In addition, nucleotide excision repair related genes (e.g., *swi10*, BC1G_09665; *SPBC582.10c*, BC1G_07679) were upregulated in cells treated with TTO, 1,8-cineole, and combined 1,8-cineole and terpinen-4-ol.

qRT-PCR analyses of selected DEGs

In order to validate the transcriptome results, we performed qRT-PCR on seven selected genes from each treatment group. As shown in Fig. 5a, c, expression of *FAS2* and *gtr-1* were downregulated and upregulated in the TTO-treated group, respectively. *GYP* expression was downregulated in terpinen-4-ol-treated and combined 1,8-cineole and terpinen-4-ol-treated cells (Fig. 5b), while expression of *ish1* was upregulated in TTO-treated, terpinen-4-ol-treated, and combined 1,8-cineole and terpinen-4-ol-treated cells (Fig. 5d). In TTO-treated cells, *yhb1* expression was downregulated, but expression was upregulated in cells treated with combined 1,8-cineole and terpinen-4-ol (Fig. 5e). Expression of *mae2* and *GPI* were downregulated in TTO-treated and combined 1,8-cineole and terpinen-4-ol-treated cells, and *GPI* was also downregulated in cells treated with 1,8-cineole (Fig. 5f, g). These results are consistent with the RNA-Seq results (Fig. 5).

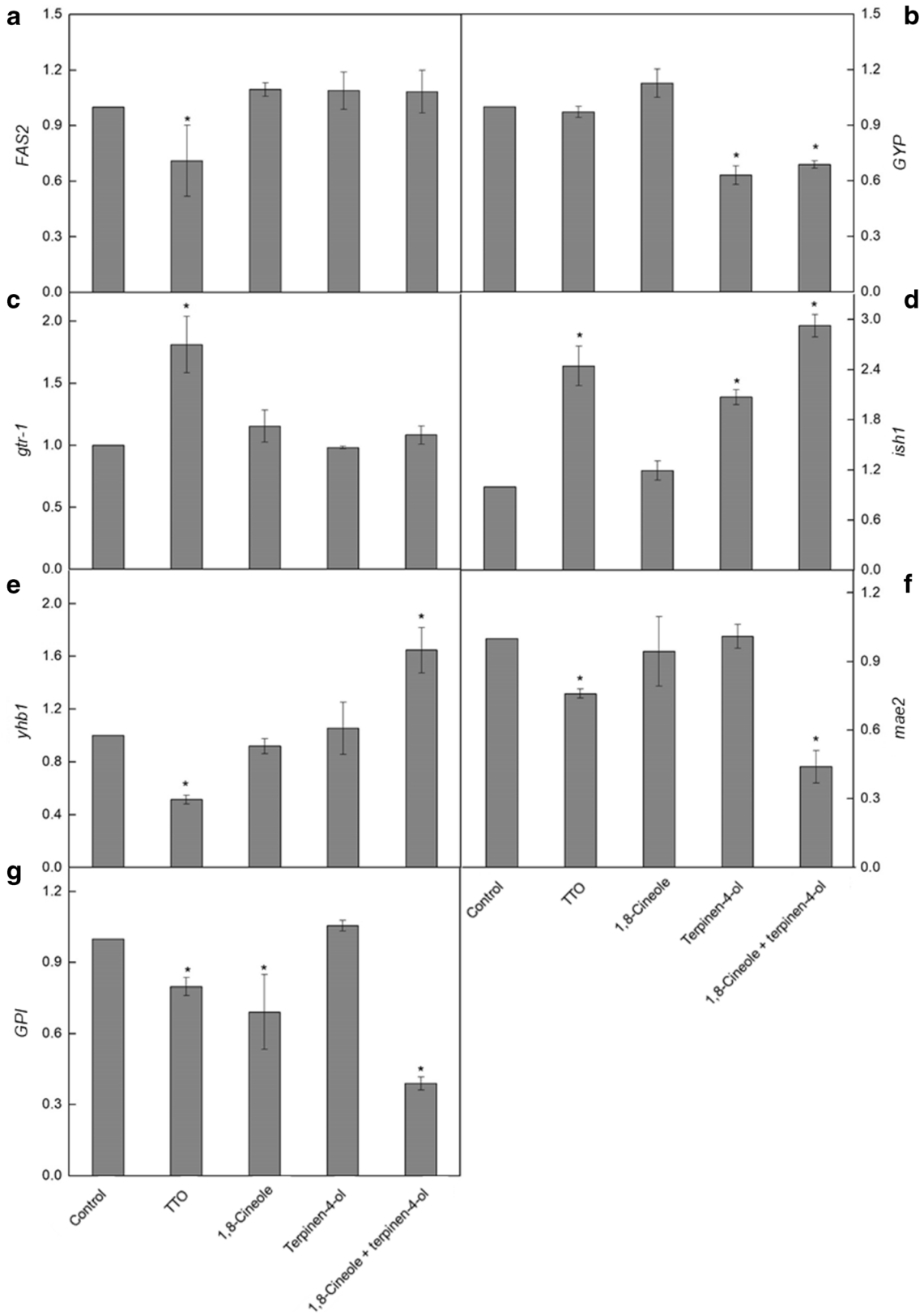

Fig. 5 Relative expression levels determined by qRT-PCR for seven selected DEGs. Asterisks indicate that expression of the DEG is significantly different ($p \leq 0.05$) from the control using ANOVA analysis followed by Duncan's multiple range tests. *FAS2* 3-oxoacyl-[acyl-carrier-protein] synthase, *GYP* hypothetical protein, *gtr-1* glutathione reductase, *ish1* stress response protein, *yhb1* flavohemoprotein, *mae2* NAD-dependent malic enzyme, *GPI* glycosylphosphatidylinositol anchor biosynthesis protein

Activity of key TCA cycle enzymes

As shown in Fig. 6a, SDH activities in *B. cinerea* cells treated with TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol were significantly ($p < 0.05$) lower than that in the control, falling to 21.4%, 67.9%, and 32.1%, respectively. Similarly, the MDH activities in cells treated with TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol were less than 13.5%, 62.2%, and 55.1% of control activities, respectively. Treatment with TTO reduced α-KGDH activity by 33.3%, but treatment with 1,8-cineole, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol caused no significant change. TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol reduced ($p < 0.05$) ICDH activity to 50.6%, 46.1%, and 47.2% of control levels, respectively. However, no significant ($p < 0.05$) change in SDH, MDH, α-KGDH, and ICDH activities was observed in response to 1,8-cineole treatment. These results suggest that treatments containing terpinen-4-ol (TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol) exert antifungal activity by obstructing the TCA cycle and disrupting mitochondrial function.

H_2O_2 accumulation and assay for CAT, POD, and SOD activities

As shown in Fig. 6b, the H_2O_2 content in *B. cinerea* cells treated with 1,8-cineole, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol increased by 58.0%, 60.4%, and 63.6% relative to the control, respectively. The highest CAT activity was observed in cells treated with combined 1,8-cineole and terpinen-4-ol, followed by cells treated with TTO and terpinen-4-ol treatment (29.4%, 21.8%, and 16.3% increases respectively). POD activities in cells treated with TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol were enhanced by 12.6%, 14.8%, and 19.0%, respectively. Interestingly, CAT and POD activities in cells treated with 1,8-cineole did not change significantly. Treatment with TTO, 1,8-cineole, and terpinen-4-ol increased SOD activity,

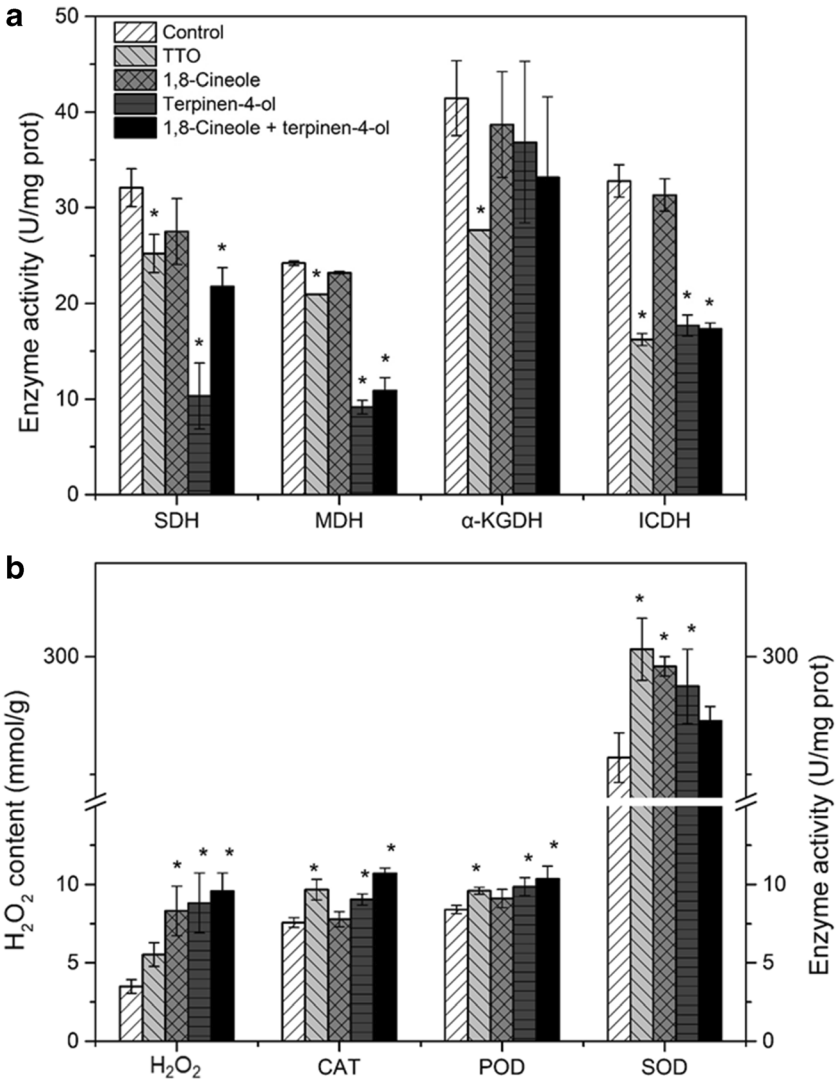

Fig. 6 Effect of TTO and its characteristic components on **a** activities of succinate dehydrogenase (SDH), malate dehydrogenase (MDH), α-ketoglutarate dehydrogenase (α-KGDH), isocitrate dehydrogenase (ICDH) and **b** catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and H_2O_2 content in *B. cinerea*

but treatment with combined 1,8-cineole and terpinen-4-ol caused no apparent increase in the activity of this enzyme. These results suggest that TTO and its two characteristic components alone and in combination induce oxidative stress in *B. cinerea* cells.

Discussion

Transcriptome analysis presents an opportunity to understand a wide variety of physiological responses by microorganisms to drugs or environmental conditions, including the fungal response to essential oils (Haridas et al. 2013; Ouyang et al. 2016; Wang et al. 2018). Most of the DEGs detected in *P. expansum* after exposure to decanal essential oils were involved in metabolism and genetic information (Zhou et al. 2018). *P. expansum* also responds to a combination of cinnamaldehyde and citral by modifying the expression of genes involved in biosynthesis of secondary metabolites, amino acid metabolism, and oxidation-reduction (Wang et al. 2018). However, high-throughput gene expression analysis has not been applied to study antifungal activities exerted by single or combined 1,8-cineole and terpinen-4-ol in essential oils. Our previous study found that terpinen-4-ol combined with 1,8-cineole has a significant synergistic effect on *B. cinerea* compared with treatment using either component alone (Yu et al. 2015). In the present study, transcriptional profiling of *B. cinerea* shows that terpinen-4-ol primarily affects the expression of genes related to biosynthesis of secondary metabolites and metabolisms of amino acid, carbohydrate, and lipid; 1,8-cineole mainly affects genes involved in genetic information processing, including transcription, replication, and repair. Thus, the synergistic antifungal effects of 1,8-cineole and terpinen-4-ol on *B. cinerea* may be due to their different actions at the transcriptional level.

Cell walls, cell membranes, mitochondria, and genetic material have been proposed as targets that explain the antifungal activity of essential oils or their volatile compositions (Bakkali et al. 2008; Shao et al. 2013a; Zheng et al. 2015; Zhou et al. 2018). The fungal cell wall is essential for sustaining cell morphology and protects the cell from mechanical damage and osmotic stress. Our previous study found that TTO caused significantly higher alkaline phosphatase activity, destroyed the cell wall, and finally ruptured the plasmalemma (Shao et al. 2013a). Changes in the expression levels of certain genes related to cell wall integrity and biosynthesis/degradation have been found in fungal cells exposed to essential oils (Brennan et al. 2013; Ouyang et al. 2016; Wang et al. 2018). In this study, five genes associated with cell wall integrity (*PIL1*, *LSP1*, *RBE1*, *MKC1*, and *gim-1*) were upregulated after exposure to terpinen-4-ol and combined 1,8-cineole and terpinen-4-ol. Genes *LSP1* and *PIL1* are involved in the negative regulation cell wall integrity signaling pathways (Zhang et al. 2004). Gene *RBE1* encodes a cell wall protein involved in cell wall integrity and plays a role in virulence (Rohm et al. 2013). Gene *MKC1*, a basic element of the cell integrity signaling pathway, is continuously activated during long-term cell surface stress in *Candida albicans*, causing expression of proteins that function in adaptation (Heilmann et al. 2013; Molero et al. 2010). Our results suggest that terpinen-4-ol and combined 1,8-cineole and terpinen-4-ol can alter the properties of *B. cinerea* cell walls while triggering a compensatory transcriptional response to cell wall damage. A similar phenomenon was observed in *Saccharomyces cerevisiae*, where limonene treatment causes the over-expression of genes involved in the cell wall integrity signaling pathway (Brennan et al. 2013).

In *Schizosaccharomyces pombe*, *EXG1* encodes a β-glucanase that participates in the metabolism of β-glucan, and *RHO2* regulates the synthesis of α-D-glucan, the other main structural polymer in the cell wall (Hirata et al. 1998). Gene *agn1* plays a role in cell separation, where it is required for the degradation of the cell wall material surrounding the septum (Dekker et al. 2004). Transcription factor Ace2p is a key regulator of cell wall metabolism in *C. albicans*, and deletion of the gene that encodes it (*CaACE2*) results in a defect in cell separation (Kelly et al. 2004). Our results showed that *EXG1*, *RHO2*, *agn1*, and *ACE2* were downregulated upon TTO treatment, indicating that TTO may block the formation of cell wall architecture and cell separation by downregulating cell wall related genes, thereby increasing the sensitivity of cells to TTO.

Given the lipophilic character of essential oils, microbial cytoplasmic membranes have been regarded as the target of essential oils or their volatile components, potentially explaining the antifungal activity of these bioactive compounds (Bakkali et al. 2008; Cabral et al. 2013; Sara 2004). Essential oils affect the expression of genes involved in cell membrane-related pathways such as fatty acid biosynthesis (*FAS1*, *FAS2*) and sterol biosynthesis (*ERG*), thereby compromising membrane integrity (Ouyang et al. 2016). The gene *FAS2* encodes the fatty acid synthase alpha subunit, which catalyzes the synthesis of long-chain saturated fatty acids. Delta (12) fatty acid desaturase is involved in the polyunsaturated fatty acid biosynthesis pathway and catalyzes the desaturation of oleic acid to linoleic acid (Sakuradani et al. 1999). We found that genes involved in fatty acid biosynthesis (*FAS2*, delta (12) fatty acid desaturase gene, and *CYP505*) were downregulated in treated cells, indicating that the composition of the cell membrane was affected by presence of TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol. Microbial cells are known to respond to environmental stress by modulating the ratio of saturated to unsaturated fatty acids (Wu et al. 2012).

Ergosterol is an essential component of fungal plasma membranes and affects membrane permeability and the

activities of membrane-bound enzymes. Transcription factors Upc2p and Ecm22p of yeast are sterol regulatory element binding proteins responsible for regulating transcription of the sterol biosynthetic genes *ERG2* and *ERG3* (Vik and Rine 2001). The gene *ATG26* encodes sterol 3-β-glucosyltransferase in the sterol biosynthetic pathway. In the present study, TTO and combined 1,8-cineole and terpinen-4-ol both caused upregulation of genes involved in sterol biosynthesis (*UPC2*, *ATG26*). *ERG* genes are upregulated in *C. albicans* cells after exposure to the antifungal drug itraconazole (De Backer et al. 2001). Our results suggest that TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol may affect membrane fluidity or permeability by changing membrane component related genes, consistent with previous observations made using fluorescent microscopy (Yu et al. 2015). In addition, we speculate that 1,8-cineole acts on different targets, possibly penetrating the cell membrane and damaging cellular organelles without causing lesions on the membrane.

Mitochondria supply energy to the cell by producing adenosine triphosphate (ATP) via the TCA cycle and oxidative phosphorylation. Mitochondria are potential targets for antifungal agents because any mitochondrial dysfunction will cause cell death (Wu et al. 2009). Citral treatment downregulates several genes (*MRPS5*, *SDH1*, *CS*, *IDH1*) involved in mitochondrial function in *P. digitatum* (Ouyang et al. 2016). Our data show that genes related to mitochondrial genetic information processing (*bI1* and *mrp51*) are downregulated, while *mmf1* is upregulated, following exposure to TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol. Gene *bI1* encodes a mitochondrial DNA endonuclease that is involved in intron homing. Gene *mrp51* encodes 37S ribosomal protein that is a component of the mitochondrial small ribosomal subunit and its deletion completely blocks mitochondrial gene expression (Greenwillms et al. 1998). The gene *mmf1* is mainly responsible for maintenance of mitochondrial DNA stability, and loss of the mitochondrial genome may occur if it is deleted (Accardi et al. 2004; Oxelmark et al. 2000). Our results suggest that treatments containing terpinen-4-ol block mitochondrial DNA processing and translation in *B. cinerea*.

Functional analysis of DEGs also indicate that some DEGs related to the response to mitochondrial dysfunction (*mae2*, *ND2*, and *BCS1*) are downregulated in cells treated with TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol. The gene *mae2* encodes NAD-dependent malic enzyme that provides mitochondrial NADH for electron transport (Artus and Edwards 1985). NADH-ubiquinone oxidoreductase chain 2 (ND2) is a subunit of complex I of the respiratory chain and encoded by the mitochondrial genome in *Chlamydomonas reinhardtii* (Cardol et al. 2002). The gene *BCS1* encodes a transmembrane chaperone of the mitochondrial inner membrane that is required for the assembly of the mitochondrial respiratory chain complex III (Koerber et al. 1998; Sawamura et al. 2014). Our results suggest that treatments containing terpinen-4-ol may cause mitochondrial dysfunction by blocking the respiration, i.e., the electron transport chain, and inhibiting the TCA cycle. A decrease in the activities of key TCA cycle-related enzymes (SDH, MDH, ICDH, and a-KGDH) was observed after exposure to TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol treatment. We speculate that treatments containing terpinen-4-ol act on mitochondria by dysregulating genes related to mitochondrial function, and bring about obstruction of the TCA cycle, ultimately resulting in cell death.

To survive drastic environmental changes, eukaryotes must rapidly adjust their gene expression programs to deploy protective mechanisms (Jian-Wu et al. 2011; Legay et al. 2011). For example, genes involved in the oxidative stress response (*CAT1*, *YBH1*, and *SOD*) are upregulated in *C. albicans* upon MAF-1 treatment (Wang et al. 2017). Gene *ish1*, which encodes a stress response protein that is present throughout the cell cycle, is induced in response to stresses such as glucose and nitrogen starvation, as well as osmotic stress (Lorena et al. 2002). Gene *yhb1* encodes flavohemoprotein, which plays a major role in resistance to nitrosative stress in *Staphylococcus aureus* (Lewis et al. 2015). Three genes (*AN8692*, *gtr-1*, and *CAT1*), which encode thiol-specific peroxidase, glutathione reductase, and catalase, respectively, are all involved in cellular response to oxidative stress. We found that stress responsive genes (*ish1*, *AN8692*, *gtr-1*, *YBH1*, and *CAT1*) were upregulated in *B. cinerea* following exposure to TTO. Among these, *ish1*, *YBH1*, and *CAT1* were also upregulated following exposure to combined 1,8-cineole and terpinen-4-ol. *ish1* was upregulated following exposure to terpinen-4-ol. Finally, H_2O_2 accumulates upon exposure to TTO and its two characteristic components, alone or in combination. This is likely to further arouse anti-oxidation systems and induce the activity of antioxidant-related enzymes (CAT, POD, SOD), consistent with the transcriptional data. Thus, our results suggest that TTO, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol all induce stress responses in *B. cinerea*.

Transcription is the first step in gene expression by which DNA templates are copied to generate RNAs (especially mRNA) by RNA polymerases. The delta subunit of RNA polymerase is required for the rapid changes in gene expression needed for cell survival under diverse stress conditions (Rabatinova et al. 2013). In *P. exansum*, treatment with decanal inhibited expression of ribosomal and aminoacyl-tRNA genes, decreasing translation capacity and inhibiting normal development (Zhou et al. 2018). In this study, genes associated with the regulation of transcription by RNA polymerase II and translational initiation were partially depressed by TTO and its two characteristic components, alone or in combination. A similar result was observed in *P. digitatum* cells treated with citral (Ouyang et al. 2016). In addition,

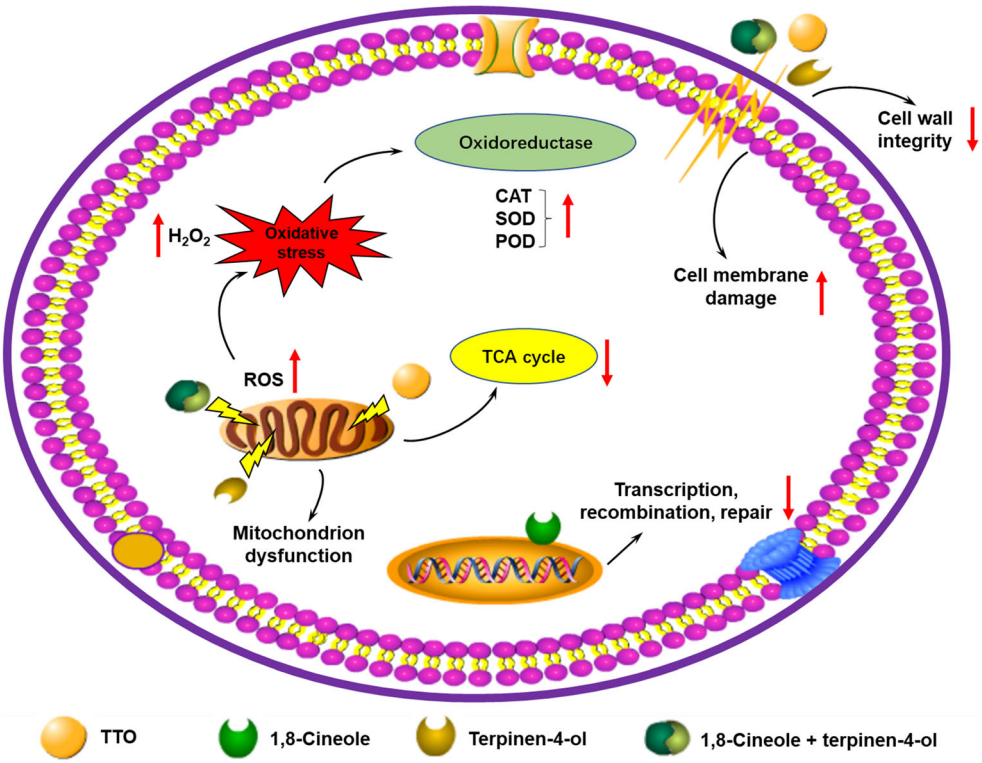

Fig. 7 Model showing mechanisms underlying the antifungal activity of TTO, 1,8-cineole, terpinen-4-ol, and combined 1,8-cineole and terpinen-4-ol against *B. cinerea*

genes playing a role in DNA replication, recombination, and repair were also repressed. Interestingly, 1,8-cineole appears to mainly influence genes involved with genetic information processing.

In conclusion, we propose a hypothetical model of action of TTO and its two characteristic components against *B. cinerea* (Fig. 7). Terpinen-4-ol exerts antifungal activity mainly by blocking the expression of genes related to cell integrity, mitochondrial function, and the respiratory chain. The resulting mitochondrial dysfunction triggers an oxidative stress response. In contrast, 1,8-cineole primarily affects genes involved in genetic information processing including DNA replication, transcription, and repair. Disrupting these essential functions ultimately induces cell death. The model accounts for the synergistic effects of terpinen-4-ol combined with 1,8-cineole, and for the antifungal activity of TTO.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain studies with human participants or animals by any of the authors.

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