

Article

Antifungal Efficacy and Mechanisms of Methyleugenol from *Asarum* Essential Oil Against *Rhizoctonia solani* Causing Ginseng Damping-Off Disease

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Abstract

Damping-off disease caused by *Rhizoctonia solani* is a devastating soilborne disease that poses a serious threat to sustainable ginseng (*Panax ginseng*) production. In an effort to develop environmentally friendly control alternatives, we investigated the volatile constituents of *Asarum heterotropoides* essential oil using GC-MS in 2023 and identified methyleugenol as the predominant bioactive component, accounting for 21.32% of the total volatile fraction. Mycelial growth rate assays demonstrated that methyleugenol exerted pronounced antifungal activity against *R. solani*, with EC₅₀ and EC₉₀ values of 60 mg/L and 800 mg/L, respectively. Treatment at 0.1 mg/mL resulted in 51% growth inhibition after 4 days of incubation. To elucidate the underlying mechanisms of action, *R. solani* mycelia exposed to 0.1 mg/mL methyleugenol were harvested at 4 and 6 days post-treatment for integrated transcriptomic and untargeted metabolomic profiling. Transcriptome analysis revealed 1444 and 1157 significantly downregulated genes at 4 and 6 days, respectively, with 836 genes consistently repressed at both time points. GO and KEGG enrichment analyses indicated that these persistently suppressed genes were predominantly associated with protein processing in the endoplasmic reticulum, protein export, tyrosine metabolism, and peroxisome biogenesis. Metabolomic profiling identified 95 differentially accumulated metabolites that were commonly reduced across both time points, with KEGG pathway enrichment highlighting significant suppression of starch and sucrose metabolism and galactose metabolism pathways central to carbon source utilization. Integrative transcriptome–metabolome correlation analysis further uncovered three key transcription factors (gene-RDB_LOCUS74145, gene-RDB_LOCUS26344, and gene-RDB_LOCUS52181) implicated in the regulation of carbohydrate metabolism and tricarboxylic acid cycle-related metabolite production. Collectively, our findings indicate that methyleugenol suppresses *R. solani* growth through a multi-target mechanism involving impairment of carbon source metabolism, disruption of protein processing and secretion, and attenuation of cellular energy supply. These results provide a mechanistic foundation for the development of botanical fungicide-based strategies for the green management of ginseng damping-off disease.



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Keywords: *Asarum heterotropoides*; plant-derived volatile constituent; soilborne fungal disease; mycelial growth inhibition; omics analysis

1. Introduction

Ginseng (*Panax ginseng* C. A. Meyer), a perennial herbaceous plant belonging to the family Araliaceae, has long been regarded as the “King of Herbs” owing to its rich repertoire of bioactive constituents, including ginsenosides, polysaccharides, and amino acids [1,2]. Pharmacological studies have demonstrated that ginseng possesses a broad spectrum of therapeutic properties, such as regulation of the central nervous system, immunomodulation, anti-fatigue, antioxidant, and antitumor activities, making it one of the most valued medicinal herbs indigenous to northeastern China [3]. At present, the cultivated area of ginseng in China exceeds 133,000 hectares, contributing over 80% of the global output, with Jilin Province alone accounting for approximately 40% of the national planting area [4].

Given its prolonged growth cycle, ginseng is highly susceptible to a wide range of diseases throughout cultivation. Among these, damping-off disease stands out as one of the most destructive soilborne diseases in ginseng production, capable of causing total crop failure under severe conditions [5]. The causal agent is *Rhizoctonia solani* Kühn, the anamorph (asexual stage) of a basidiomycete belonging to the genus *Rhizoctonia*, which is widely recognized as one of the most damaging soilborne phytopathogens worldwide [6]. This fungus overwinters in soil as mycelia and sclerotia, and upon favorable conditions in the following spring, infects the roots and stem bases of ginseng seedlings, producing water-soaked brown lesions that frequently lead to seedling collapse and death [7]. Current management of ginseng damping-off relies predominantly on synthetic fungicides such as hymexazol and thiophanate-methyl [8]. However, prolonged and intensive application of chemical agents has raised serious concerns, including the emergence of fungicide-resistant pathogen populations, pesticide residue accumulation, and disruption of soil microbial communities [9,10]. Consequently, the development of highly effective, low-toxicity, and environmentally benign botanical fungicides has become a priority in the green management of ginseng diseases.

Botanical fungicides, whose active ingredients are derived from natural plants, have attracted increasing attention in recent years due to their environmental compatibility, low risk of resistance development, and biodegradability [11,12]. Several plant-derived compounds and essential oils have been reported to inhibit *R. solani*. For example, eugenol was found to suppress *R. solani* through actions on multiple cellular targets [13], and a number of essential oils and their major constituents, such as cinnamaldehyde, carvacrol, and thymol, have shown inhibitory effects on the mycelial growth of this pathogen [14]. These studies indicate that botanical agents are a feasible option for the control of *Rhizoctonia* diseases. However, most previous work has focused on the evaluation of antifungal activity, whereas the molecular mechanisms involved, particularly those revealed by combined transcriptomic and metabolomic analyses, have rarely been examined.

Asarum spp. (Aristolochiaceae) are perennial herbaceous plants with a long history of medicinal use in China. These species are widely distributed across northeastern China and share overlapping ecological niches with ginseng [15]. Previous studies have revealed that *Asarum* possesses notable antimicrobial properties, with its essential oil exhibiting inhibitory effects against various phytopathogenic fungi [16,17]. Methyleugenol (4-allyl-1,2-dimethoxybenzene), one of the principal bioactive constituents of *Asarum* volatile oil, can reach a relative content as high as 25.67% [18]. This compound has been reported to

exhibit insecticidal, nematocidal, and antifungal activities [19,20]. Our preliminary work demonstrated that methyleugenol is effective in controlling soil-dwelling nematodes [21], suggesting its potential utility against soilborne fungal pathogens. Nevertheless, the antifungal activity of methyleugenol against *R. solani* and the molecular mechanisms underlying its inhibitory action remain poorly characterized.

Transcriptomics and metabolomics enable comprehensive elucidation of molecular responses at the gene expression and metabolite levels under defined conditions [22,23]. Transcriptomic profiling captures genome-wide gene expression patterns at specific developmental stages and has been extensively employed to dissect molecular mechanisms governing diverse biological processes [24]. Metabolomics, by contrast, monitors changes in small-molecule metabolites and thereby provides a direct readout of phenotypic status and metabolic activity [25]. The integration of these two approaches offers a “gene-to-metabolite” perspective for deciphering complex regulatory networks and has emerged as a powerful strategy for unraveling the modes of action of bioactive compounds against fungal pathogens [26,27].

Against this background, the present study was designed to: (1) systematically evaluate the inhibitory efficacy of methyleugenol on *R. solani* mycelial growth; (2) employ an integrated transcriptomic and metabolomic approach to delineate the molecular mechanisms by which methyleugenol suppresses *R. solani* at both the transcriptional and metabolic levels; and (3) identify core genes and critical metabolic pathways associated with fungal inhibition, thereby providing a theoretical basis for the development of botanical fungicides and green control strategies against ginseng damping-off disease.

2. Materials and Methods

2.1. Materials

2.1.1. Fungal Strain

The *Rhizoctonia solani* strain RCC5 used in this study was originally isolated from the roots of diseased one-year-old ginseng plants collected from a ginseng plantation in Changchun, Jilin Province, China, in 2011. The strain was purified and confirmed as the causal agent of ginseng damping-off according to Koch’s postulates. It was subsequently identified as a multinucleate *Rhizoctonia* on the basis of morphological characteristics and nuclear staining, and assigned to *R. solani* anastomosis group AG-5 through hyphal anastomosis testing. Molecular identification based on the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) further confirmed this classification, and the sequence has been deposited in GenBank under accession number HF912167. The strain was maintained by the Laboratory of Integrated Plant Disease Management, Jilin Agricultural University, Changchun, China [28].

2.1.2. Plant Material

Roots of five-year-old *Asarum heterotropoides* Fr. Schmidt were obtained from a medicinal-plant cultivation base in Songjianghe Town, Fusong County, Baishan City, Jilin Province, China, in 2023. The cultivated plants had been domesticated from wild germplasm originally collected in the surrounding mountainous areas; as no registered cultivar is currently available for this species, no cultivar name is assigned. The material was identified as *A. heterotropoides* based on its morphological characteristics, in accordance with the descriptions in the Flora of China and the Pharmacopoeia of the People’s Republic of China. The plants were perennial herbs with a horizontally growing rhizome bearing numerous slender, densely fascicled roots that emitted a characteristic pungent aroma. Each shoot bore two cordate to reniform-cordate leaves with a deeply cordate base and an acute to shortly acuminate apex. Solitary, purplish-brown, bell- to cup-shaped flowers arose from between the leaf bases, with three triangular-ovate perianth (calyx) lobes that

spread horizontally and were abruptly reflexed against the perianth tube [29]. These features were consistent with the typical characteristics of the species. The identification was carried out by a professor specializing in the cultivation and domestication of wild medicinal plants at the Institute of Chinese Medicinal Materials, Jilin Agricultural University, Changchun, China.

2.1.3. Reagents

The Total RNA Rapid Extraction Kit was purchased from Novogene Co., Ltd. (Beijing, China). Methyleugenol (purity $\geq 98\%$, Cat. No. S25645) was obtained from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). The LC–MS-grade solvents and reagents used in this study included methanol (CAS 67-56-1, Cat. No. A456-4; Thermo Fisher Scientific, Waltham, MA, USA), ultrapure water (CAS 7732-18-5, Cat. No. 1.15333.2500; Merck, Darmstadt, Germany), formic acid (CAS 64-18-6, Cat. No. A117-50; Thermo Fisher Scientific, Waltham, MA, USA), and ammonium acetate (CAS 631-61-8, Cat. No. A114-50; Thermo Fisher Scientific, Waltham, MA, USA). All other chemicals were of analytical grade and domestically sourced.

2.1.4. Instruments

The major instruments employed in this study included a Q Exactive™ HF-X hybrid quadrupole-Orbitrap mass spectrometer (Thermo Fisher, Dreieich, Germany), a Vanquish UHPLC system (Thermo Fisher, Dreieich, Germany), a Hypersil Gold column (100 mm $\times$ 2.1 mm, 1.9 μ m; Thermo Fisher, Waltham, MA, USA), a D3024R refrigerated microcentrifuge (Scilogex, Rocky Hill, CT, USA), a ZEISS SERIES 120 microscope (Shanghai Chaotan Materials Co., Ltd., Shanghai, China), a BSA124S-CW analytical balance (Sartorius, Göttingen, Germany), an SW-CJ-2FD laminar flow clean bench (Suzhou Antai, Suzhou, China), an XFH-40CA vertical autoclave (Shanghai Gaozhi Precision Instruments, Shanghai, China), a DK-S24 thermostatic water bath (Shanghai Jinghong, Shanghai, China), and a DHP-9082 constant-temperature incubator (Ningbo Haishu Saifu, Ningbo, China).

2.2. Chemical Profiling of *Asarum* Essential Oil

Volatile oil was extracted from *Asarum* roots by steam distillation following previously described protocols [30]. Briefly, freshly collected root material was pulverized and placed into a volatile oil extraction apparatus. Distilled water was added at a solid-to-liquid ratio of 1:10 (*w/v*), and reflux distillation was carried out for 6 h to collect the volatile oil fraction. The chemical composition of the resulting volatile oil was analyzed by gas chromatography–mass spectrometry (GC-MS). Individual components were identified by comparison of their mass spectra with entries in the NIST database, and relative contents were determined using the peak area normalization method.

2.3. Evaluation of the Antifungal Activity of Methyleugenol Against *R. solani*

2.3.1. Preparation of Amended Media

Potato dextrose agar (PDA) medium was sterilized by autoclaving at 121 °C for 30 min. Methyleugenol was dissolved in acetone containing 0.1% (*v/v*) Tween-80 and serially diluted. Appropriate volumes of each dilution were incorporated into molten PDA cooled to approximately 50 °C to yield final methyleugenol concentrations of 1, 0.2, 0.1, 0.05, 0.02, and 0.01 mg/mL. Unamended PDA plates served as the negative control. Three replicate plates were prepared for each treatment.

2.3.2. Determination of Mycelial Growth Inhibition

Mycelial plugs (8.0 mm in diameter) were excised from the actively growing margin of 5-day-old *R. solani* colonies using a sterile cork borer and transferred to the center of

each amended plate. Inoculated plates were incubated at 25 °C in a constant-temperature incubator. Colony diameters were measured using the cross-intersection method at 4, 6, and 8 days after inoculation. The percentage of mycelial growth inhibition was calculated according to the following formula:

$$\text{Inhibition rate (\%)} = [(D_c - D_t) / (D_c - d)] \times 100$$

D_c is the colony diameter of the control, D_t is the colony diameter of the treatment, and d is the diameter of the initial mycelial plug (8.0 mm) [31].

The effective concentrations causing 50% and 90% inhibition of mycelial growth (EC_{50} and EC_{90}) were estimated by probit regression analysis of inhibition rates against the logarithm of methyleugenol concentrations using SPSS 26.0 software (IBM Corp., Armonk, NY, USA).

2.4. Sample Preparation for Transcriptomic and Metabolomic Analyses

Potato dextrose (PD) liquid medium was autoclaved at 121 °C for 20 min. Methyleugenol was dissolved in acetone containing 0.1% (*v/v*) Tween-80 and added to the sterilized PD medium to achieve a final concentration of 0.1 mg/mL. Mycelial plugs (8.0 mm diameter) from actively growing *R. solani* colonies were inoculated into 250 mL glass Erlenmeyer flasks containing the methyleugenol-amended medium. Flasks containing unamended PD medium inoculated with identical mycelial plugs served as controls. All flasks were incubated at 25 °C on a rotary shaker at 150 rpm.

The sampling time points were determined based on the EC_{50} value, actual mycelial growth observations, and the minimum biomass requirements for sequencing. Samples were collected at day 4, when the accumulated mycelial biomass in the treatment group first met the minimum threshold for transcriptome sequencing, and at day 6, when growth differences between treated and control groups were highly significant. Upon harvest, mycelial samples were immediately flash-frozen in liquid nitrogen, transported to the laboratory, and stored at −80 °C until further processing. Three independent biological replicates were prepared for each group.

The experimental design comprised four groups: Group A—control at 4 days (LZD_CK); Group B—methyleugenol-treated at 4 days (LZD); Group C—control at 6 days (LZF_CK); Group D—methyleugenol-treated at 6 days (LZF).

2.5. Transcriptome Sequencing and Analysis

2.5.1. RNA Extraction and Quality Assessment

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Beijing, China). RNA integrity was first examined by agarose gel electrophoresis. RNA concentration and purity were determined with a NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was further evaluated as the RIN value using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

2.5.2. Library Construction and Sequencing

mRNA was enriched from total RNA using oligo(dT) magnetic beads and randomly fragmented into approximately 200 bp fragments with divalent cations in NEB Fragmentation Buffer. Using the fragmented mRNA as a template and random oligonucleotides as primers, first-strand cDNA was synthesized with M-MuLV reverse transcriptase. The RNA strand was then degraded by RNase H, and second-strand cDNA was synthesized using DNA Polymerase I with dNTPs as substrates. After purification, the double-stranded cDNA underwent end repair, A-tailing, and adapter ligation. Fragments of 250–300 bp

were selected with AMPure XP beads, followed by PCR amplification and purification to generate the final library.

Library concentration was initially quantified with a Qubit 2.0 Fluorometer, the insert size was assessed on an Agilent 2100 Bioanalyzer, and the effective concentration was accurately determined by qRT-PCR (>1.5 nM). Qualified libraries were sequenced on an Illumina HiSeq platform.

2.5.3. Bioinformatic Analysis

Raw reads were quality-filtered using fastp by removing reads containing adapter sequences, those with more than 10% ambiguous bases (N), and those in which low-quality bases accounted for more than 50% of the read, yielding clean reads. Clean reads were aligned to the *Rhizoctonia solani* reference genome using HISAT2, and gene expression levels were quantified as FPKM (fragments per kilobase of transcript per million mapped fragments).

Differentially expressed genes (DEGs) were identified with DESeq2 using thresholds of $|\log_2\text{FoldChange}| > 1$ and an adjusted p -value (padj) < 0.05 . Gene Ontology (GO) functional enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses of the DEGs were performed using clusterProfiler, with $\text{padj} < 0.05$ set as the threshold for significant enrichment.

2.6. Metabolomic Analysis

2.6.1. Metabolite Extraction

A 1 mL aliquot of mycelial sample from each group was lyophilized in a freeze-dryer. Subsequently, 100 μL of 80% aqueous methanol was added, and the mixture was vortexed and then incubated on ice for 5 min. After centrifugation at $15,000 \times g$ for 15 min at 4°C , the supernatant was collected and diluted with LC-MS-grade water to a final methanol concentration of 53%. The sample was centrifuged again at $15,000 \times g$ for 15 min at 4°C , and the resulting supernatant was subjected to LC-MS analysis.

Quality control (QC) samples were prepared by pooling equal volumes of all experimental samples. Blank samples were prepared using 53% aqueous methanol in place of the experimental samples and were processed following the same protocol.

2.6.2. Chromatographic Conditions

Chromatographic separation was performed on a Hypersil Gold column (C18, 100 mm $\times$ 2.1 mm, 1.9 μm) maintained at 40°C , with a flow rate of 0.2 mL/min. For positive ion mode, mobile phase A was 0.1% aqueous formic acid and mobile phase B was methanol. For negative ion mode, mobile phase A was 5 mM ammonium acetate (pH 9.0) and mobile phase B was methanol. The gradient elution program is detailed in Table 1.

Table 1. Chromatographic gradient elution program.

Time (min)	Mobile Phase A (%)	Mobile Phase B (%)
0	98	2
1.5	98	2
3	15	85
10	0	100
10.1	98	2
11	98	2
12	98	2

Note: Mobile phases A and B were defined as follows: in positive ion mode, mobile phase A was 0.1% aqueous formic acid and mobile phase B was methanol; in negative ion mode, mobile phase A was 5 mM ammonium acetate (pH 9.0) and mobile phase B was methanol. A (%) and B (%) denote the volumetric proportions of mobile phases A and B, respectively.

2.6.3. Mass Spectrometric Conditions

Mass spectra were acquired over a scan range of m/z 100–1500. The electrospray ionization (ESI) source parameters were as follows: spray voltage, 3.5 kV; sheath gas flow rate, 35 psi; auxiliary gas flow rate, 10 L/min; ion transfer tube temperature, 320 °C; S-lens RF level, 60; and auxiliary gas heater temperature, 350 °C. Both positive and negative ion modes were employed. MS/MS spectra were acquired using data-dependent scans.

2.6.4. Data Processing and Metabolite Identification

Raw data were processed using Compound Discoverer 3.3 (CD3.3) software for peak alignment, peak extraction, and peak area quantification. Metabolites were identified by matching against the mzCloud and mzVault high-resolution MS/MS spectral databases and the MassList primary database, with a mass tolerance of 5 ppm. Background ions were removed based on the blank samples, and the raw quantitative results were normalized. Compounds with a coefficient of variation (CV) > 30% in the QC samples were excluded, yielding the final metabolite identification and relative quantification results.

2.6.5. Screening of Differential Metabolites and Statistical Analysis

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed using metaX software to obtain the variable importance in projection (VIP) value for each metabolite. The statistical significance (p value) of each metabolite between the two groups was determined by t -tests, and the fold change (FC) was calculated. Differential metabolites were identified using the following criteria: $VIP > 1$, $p < 0.05$, and $FC \geq 2$ or $FC \leq 0.5$. KEGG pathway enrichment analysis of the differential metabolites was conducted, with $p < 0.05$ considered to indicate significant enrichment.

2.7. Integrated Transcriptomic and Metabolomic Analysis

An integrated analysis was conducted on the differentially expressed genes (DEGs) and differential metabolites (DMs) from the B/A and D/C comparison groups. Correlations between DEGs and DMs were assessed using Pearson correlation coefficients ($p < 0.05$ considered statistically significant). Correlation coefficients were calculated using the `cor()` function in R, and correlation network diagrams were generated using the `corrplot` package. Particular attention was given to the regulatory relationships between genes consistently downregulated and metabolites consistently reduced across both time points, in order to elucidate the core regulatory network underlying the inhibition of *R. solani* by methyleugenol.

2.8. Statistical Analysis

All experiments were performed with at least three biological replicates, and data are presented as the mean $\pm$ standard deviation (mean $\pm$ SD). Statistical analyses were performed using SPSS 26.0 software. Significant differences were evaluated by one-way analysis of variance (ANOVA) followed by Tukey's honest significant difference (HSD) test, with $p < 0.05$ considered statistically significant.

3. Results

3.1. Analysis of Volatile Oil Components from Asarum Roots

GC-MS analysis identified multiple bioactive constituents in the volatile oil extracted from Asarum roots (Table 2). Among these, methyleugenol exhibited the highest relative content (21.32%) and was identified as the predominant active component, followed by safrole and asarone. Given its high abundance in the Asarum volatile oil and its previously reported antifungal potential, methyleugenol was selected as the primary focus of this study.

Table 2. Analysis results of chemical constituents of volatile oil of *Asarum heterotropoides* by GC-MS.

No.	Name	Molecular Formula	CAS No.	Mean Relative Content (%)
1	Methyl eugenol	C ₁₁ H ₁₄ O ₂	93-15-2	21.32 ± 0.08
2	α-Linolenic acid	C ₁₈ H ₃₀ O ₂	463-40-1	10.66 ± 0.54
3	Ethyl linoleate	C ₂₀ H ₃₆ O ₂	544-35-4	9.64 ± 0.16
4	α-Pinene	C ₁₀ H ₁₆	80-56-8	8.18 ± 0.08
5	α-Asarone	C ₁₂ H ₁₆ O ₃	2883-98-9	7.51 ± 0.30
6	Elemicin	C ₁₂ H ₁₆ O ₃	487-11-6	5.51 ± 0.15
7	Myristicin	C ₁₁ H ₁₂ O ₃	607-91-0	5.30 ± 0.19
8	Palmitic acid	C ₁₆ H ₃₂ O ₂	57-10-3	4.97 ± 0.19
9	Oleic Acid	C ₁₈ H ₃₄ O ₂	112-80-1	3.89 ± 0.24
10	Safrole	C ₁₀ H ₁₀ O ₂	94-59-7	1.33 ± 0.03
11	β-Pinene	C ₁₀ H ₁₆	127-91-3	1.21 ± 0.03
12	3,5-Dimethoxytoluene	C ₉ H ₁₂ O ₂	4179-19-5	0.88 ± 0.01
13	β-Myrcene	C ₁₀ H ₁₆	123-35-3	0.78 ± 0.01
14	Methyl isoeugenol	C ₁₁ H ₁₄ O ₂	6380-24-1	0.67 ± 0.02
15	D-Limonene	C ₁₀ H ₁₆	5989-27-5	0.58 ± 0.01

3.2. Antifungal Activity of Methyleugenol Against *R. solani*

The inhibitory effects of methyleugenol against *Rhizoctonia solani* at different concentrations are presented in Table 3 and Figures S1–S3. Methyleugenol exhibited a marked, concentration-dependent inhibitory effect on *R. solani* isolated from ginseng, with greater inhibition observed at higher concentrations. After 4 days of incubation, the inhibition rates at 0.01, 0.05, and 0.1 mg/mL were 19.18%, 31.41%, and 50.82%, respectively. After 6 days, the corresponding rates were 14.00%, 29.06%, and 48.94%, and after 8 days, 12.00%, 18.00%, and 22.00%, respectively.

Table 3. Inhibitory effects of methyleugenol against *R. solani* at different concentrations and incubation times.

Concentration (mg/mL)	Incubation for 4 d		Incubation for 6 d		Incubation for 8 d	
	Inhibition Rate (%)	Colony Diameter (cm)	Inhibition Rate (%)	Colony Diameter (cm)	Inhibition Rate (%)	Colony Diameter (cm)
0.01	19.18	6.87 ^b ± 0.42	14.00	7.31 ^d ± 0.22	12.00	7.48 ^c ± 0.09
0.05	31.41	5.83 ^b ± 1.04	29.06	6.03 ^c ± 0.18	18.00	6.97 ^b ± 0.12
0.10	50.82	4.18 ^c ± 0.42	48.94	4.34 ^b ± 0.04	22.00	6.63 ^b ± 0.06
ck	--	8.50 ^a ± 0	--	8.50 ^a ± 0	--	8.50 ^a ± 0

Note: “--” means no inhibition. Values are presented as means ± standard deviation. Different letters within the same column indicate significant differences among treatments according to Tukey’s test ($p < 0.05$).

Across all time points, methyleugenol at 0.10 mg/mL showed the strongest inhibition of *R. solani*. As incubation progressed, the inhibition rate initially remained stable and then declined, with the most pronounced effect occurring between 4 d and 6 d. Toxicity regression analysis yielded EC₅₀ and EC₉₀ values of 60 mg/L and 800 mg/L, respectively.

3.3. Transcriptome Sequencing Data Quality and Overall Analysis

3.3.1. Assessment of Sequencing Data Quality

A summary of the transcriptome sequencing data quality is provided in Table 4. Each sample yielded more than 6 G of clean data, with Q20 and Q30 values exceeding 96.64% and 91.47%, respectively, and GC content ranging from 52.75% to 54.13%. These results indicate high sequencing quality, meeting the requirements for subsequent analyses.

Table 4. Summary of transcriptome sequencing data quality.

Sample	Clean_Reads	Clean_Bases (G)	Error_Rate (%)	Q20 (%)	Q30 (%)	GC Content (%)
LZD_CK_1	43,458,588	6.52 G	0.03	97.37	93.03	53.57
LZD_CK_2	48,596,416	7.29 G	0.02	98.38	95.27	53.66
LZD_CK_3	42,786,956	6.42 G	0.03	97.53	93.4	54.13
LZD_1	45,256,630	6.79 G	0.03	97.58	93.36	52.75
LZD_2	47,532,222	7.13 G	0.03	96.64	91.47	53.42
LZD_3	43,867,922	6.58 G	0.03	97.41	93.15	53.24
LZF_CK_1	41,278,478	6.19 G	0.03	97.49	93.29	52.96
LZF_CK_2	43,903,170	6.59 G	0.03	97.4	93.05	53.57
LZF_CK_3	45,874,648	6.88 G	0.03	97.46	93.18	53.6
LZF_1	43,225,198	6.48 G	0.03	97.29	92.84	53.63
LZF_2	43,534,262	6.53 G	0.03	97.48	93.24	53.38
LZF_3	42,667,564	6.4 G	0.03	97.51	93.33	53.52

Note: LZD and LZF denote *R. solani* mycelial samples collected at 4 and 6 days post-treatment, respectively; CK, untreated control (check), i.e., mycelia cultured without methyleugenol; the numerals 1–3 indicate three biological replicates. Clean reads, the number of high-quality reads remaining after removal of adapters and low-quality reads; clean bases, the total number of bases contained in the clean reads (G, gigabases); error rate, the average base-calling error rate of the sequencing; Q20, the percentage of bases with a Phred quality score ≥ 20 (corresponding to a base-call accuracy $\geq 99\%$); Q30, the percentage of bases with a Phred quality score ≥ 30 (corresponding to a base-call accuracy $\geq 99.9\%$); GC content, the percentage of guanine (G) and cytosine (C) bases among the total bases of the clean reads.

3.3.2. Inter-Sample Correlation and Principal Component Analysis

Evaluation of transcriptome data quality and sample clustering is shown in Figure 1. Figure 1A shows the heatmap of Pearson correlation coefficients among all samples; both the horizontal and vertical axes represent the individual samples, and the color scale (R^2) indicates the square of the Pearson correlation coefficient between each pair of samples, with darker colors denoting higher correlation. Figure 1B shows the principal component analysis (PCA) score plot; the abscissa and ordinate correspond to the first (PC1, 39.15%) and second (PC2, 13.90%) principal components, respectively, and the percentages indicate the proportion of total variance explained by each component.

Correlation analysis (Figure 1A) revealed mean intra-group coefficients of determination (R^2) exceeding 0.95 for the two control groups (LZD_CK and LZF_CK) and exceeding 0.90 for the two treatment groups (LZD and LZF), indicating good reproducibility among biological replicates and high data reliability.

Principal component analysis (PCA; Figure 1B) showed clear separation between the methyleugenol-treated groups (LZD and LZF) and their respective controls (LZD_CK and LZF_CK) in the principal component space. A significant difference was also observed between the 4-day (LZD) and 6-day (LZF) treatment groups, whereas the 4-day (LZD_CK) and 6-day (LZF_CK) controls did not differ appreciably. These findings indicate that methyleugenol treatment markedly altered the gene expression profile of *R. solani*.

3.4. Analysis of Differentially Expressed Genes

3.4.1. Identification of Differentially Expressed Genes

Volcano plot analysis (Figure 2) revealed substantial changes in gene expression following methyleugenol treatment compared with the controls. A total of 2861 DEGs were identified after 4 days of treatment (B/A), comprising 1417 upregulated and 1444 downregulated genes, whereas 1954 DEGs were detected after 6 days (D/C), including 797 upregulated and 1157 downregulated genes (Figure 3A).

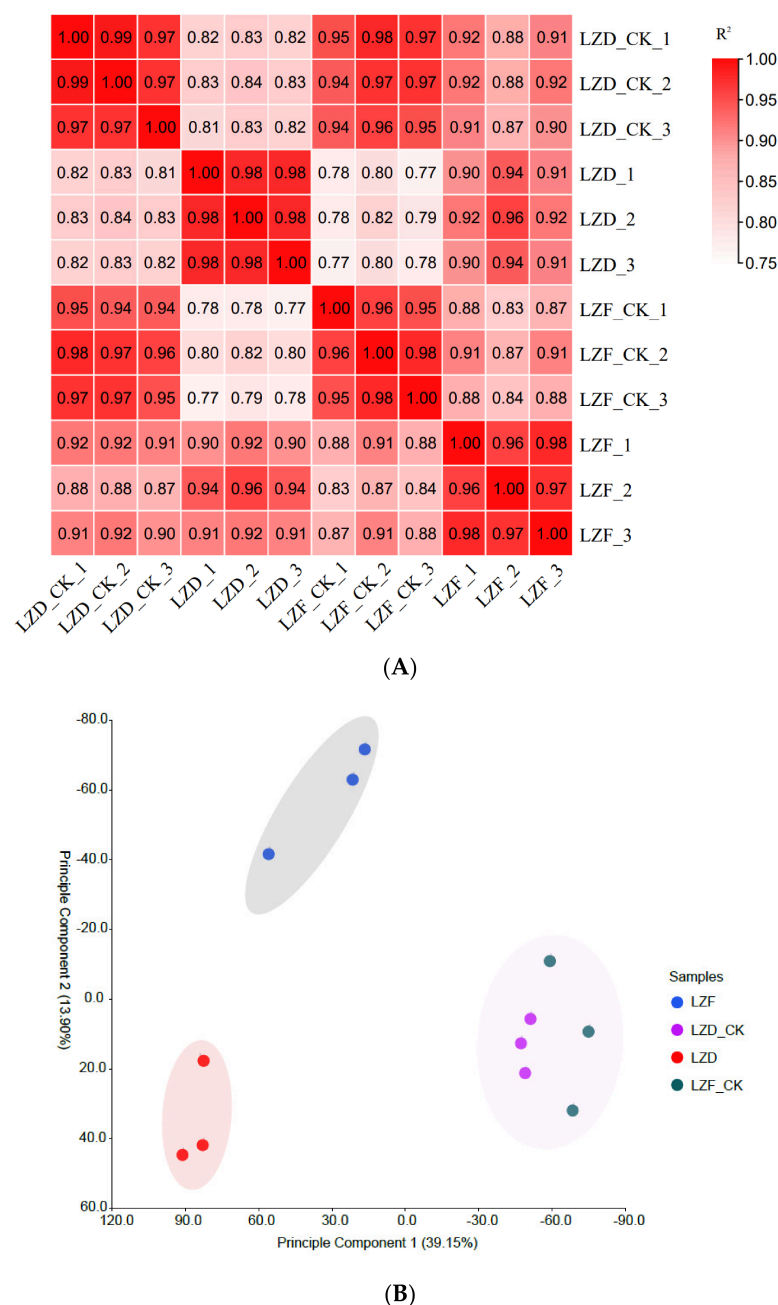


Figure 1. (A) Heatmap of inter-sample correlations. Note: The value in each cell represents the coefficient of determination (R^2 , the square of the Pearson correlation coefficient) between two samples. (B) Principal component analysis results. Note: LZD, *R. solani* mycelia treated with methyleugenol for 4 days; LZD, *R. solani* mycelia treated with methyleugenol for 6 days; LZD_CK and LZD_CK, the corresponding untreated controls (check) cultured without methyleugenol at 4 and 6 days, respectively; the suffix numerals 1–3 denote the three independent biological replicates of each group.

3.4.2. Hierarchical Clustering of Differentially Expressed Genes

Hierarchical clustering of all DEGs (Figure 4) showed minimal differences in gene expression between the 4-day and 6-day controls, in contrast to the pronounced changes observed between the corresponding treatment groups. This pattern further confirmed the marked disruptive effect of methyleugenol on the gene expression of *R. solani*.

3.5. GO and KEGG Enrichment Analyses of Differentially Expressed Genes

3.5.1. Functional Enrichment of DEGs After 4 Days of Treatment

GO enrichment analysis (Figure 5A) showed that the B/A DEGs were primarily enriched in the molecular function (MF) and cellular component (CC) categories. Within the MF category, significantly enriched terms included metal ion binding, iron ion binding, hydrolase activity, and oxidoreductase activity. KEGG pathway enrichment analysis (Figure 5B) indicated that the DEGs were mainly enriched in pathways such as protein processing in the endoplasmic reticulum and protein export.

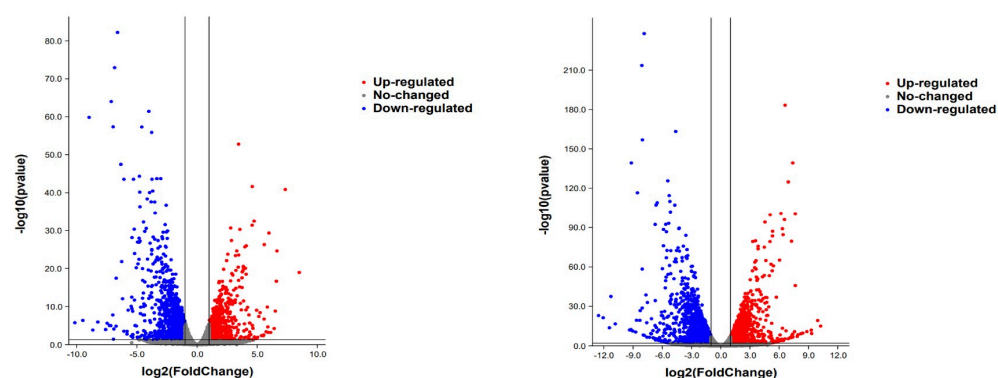
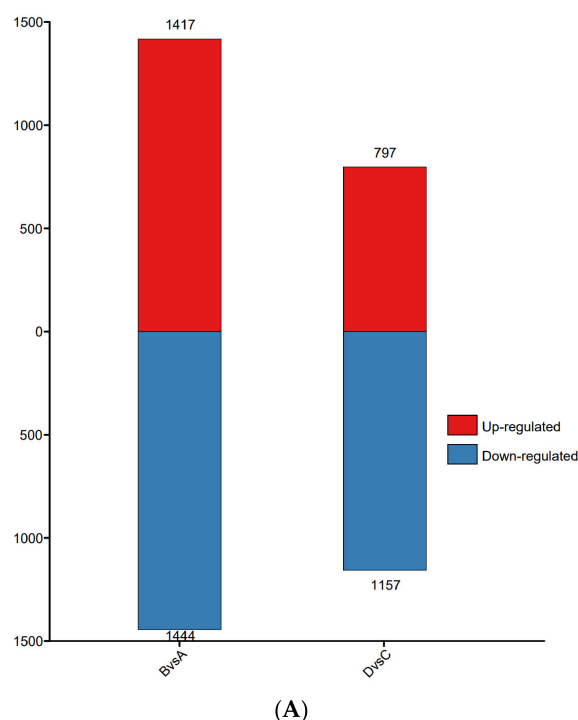


Figure 2. Volcano diagram of differential genes. Note: The abscissa is the $\log_2$ Fold Change value, the ordinate is $-\log_{10}$ padj or $-\log_{10}$ p -value, and the blue dotted line represents the threshold line of the differential gene screening criteria.



(A)

Figure 3. Cont.

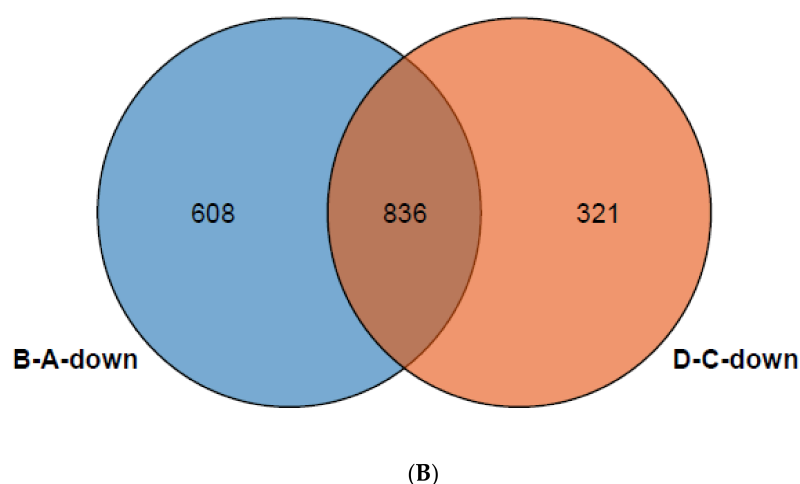


Figure 3. (A) Statistical analysis of differential genes between 4-day and 6-day treatment with methyleugenol and CK in *R. solani*. Venn diagram analysis (B) identified 836 genes that were downregulated at both time points, suggesting sustained suppression by methyleugenol. These genes may constitute the core set of target genes underlying its antifungal activity. (B) Common and unique differentially expressed genes downregulated compared to the CK group after treatment with 4 d and 6 d.

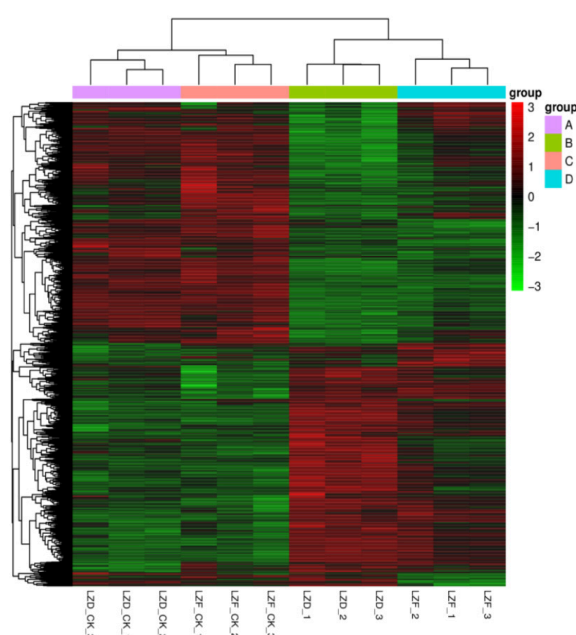


Figure 4. Heat map of differentially expressed gene clustering. Note: The abscissa is the sample name, and the ordinate is the normalized value of the differential gene FPKM, the redder the color, the higher the expression level, and the greener the expression level.

3.5.2. Functional Enrichment of DEGs After 6 Days of Treatment

GO analysis (Figure 6A) revealed that the D/C DEGs were predominantly enriched in the MF category, including iron ion binding, oxidoreductase activity, heme binding, and cofactor binding.

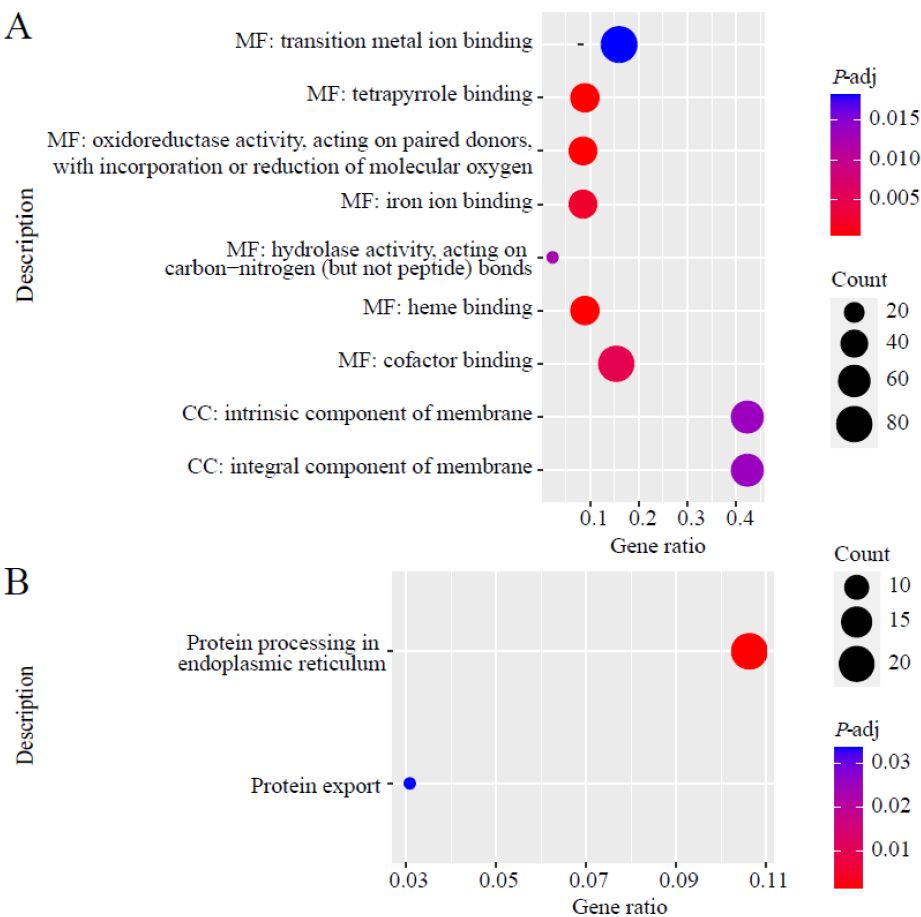


Figure 5. GO and KEGG enrichment analysis of differentially expressed genes between the methyl eugenol treated group and the control group at 4 days post-treatment. **(A)** GO enrichment analysis of differentially expressed genes; **(B)** KEGG enrichment analysis of differentially expressed genes.

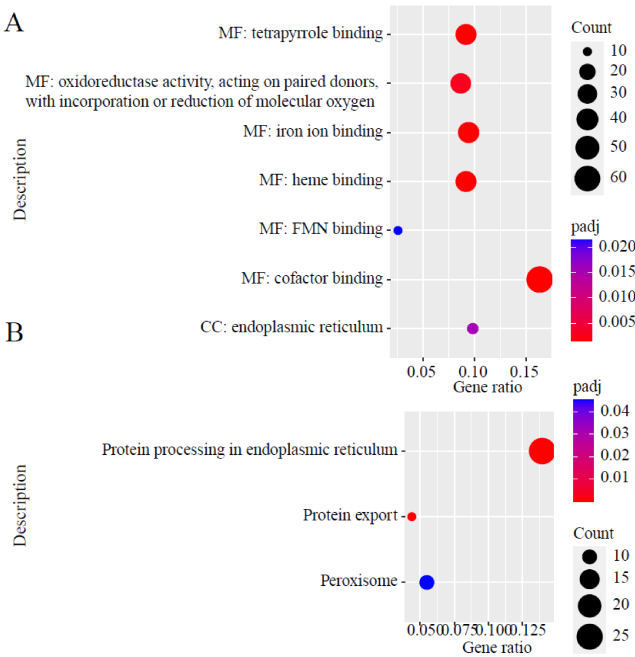


Figure 6. GO and KEGG enrichment analysis of differentially expressed genes between the methyl eugenol treated group and the control group at 6 days post-treatment. **(A)** GO enrichment analysis of differentially expressed genes; **(B)** KEGG enrichment analysis of differentially expressed genes.

KEGG pathway enrichment analysis (Figure 6B) demonstrated significant enrichment in pathways including protein processing in the endoplasmic reticulum, protein export, and peroxisome.

3.5.3. Functional Enrichment of Genes Commonly Downregulated at Both Time Points

Functional enrichment analysis of the 836 genes downregulated in both the B/A and D/C comparisons (Figure 7) yielded the following results.

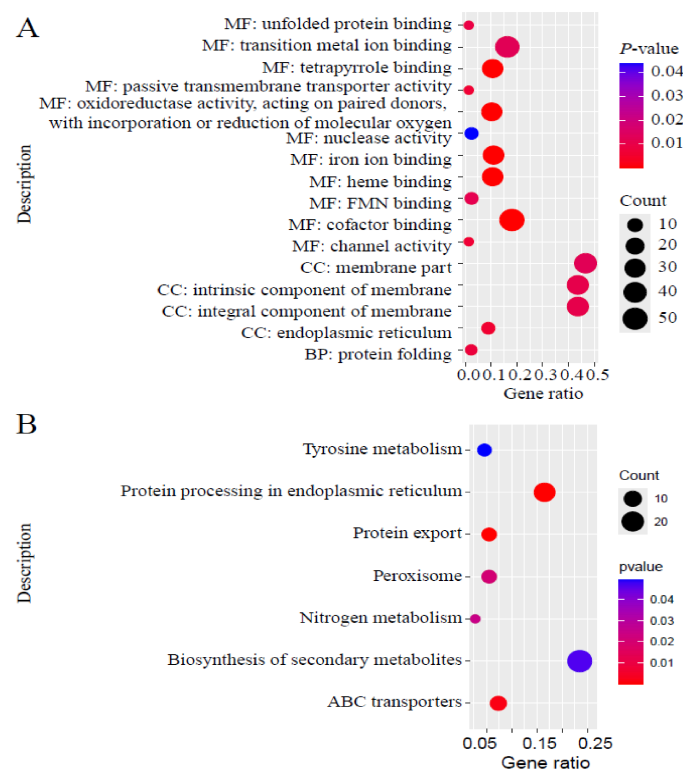


Figure 7. GO and KEGG enrichment analysis of genes that are down-regulated in both time periods following treatment with eugenol. (A) GO enrichment analysis of differentially expressed genes; (B) KEGG enrichment analysis of differentially expressed genes.

Within GO_MF (Figure 7A), functions associated with metal ion binding (including iron ion binding), heme binding, cofactor binding, and oxidoreductase activity were significantly downregulated. Within GO_CC, the plasma membrane, intrinsic components of the membrane, and transmembrane proteins were also significantly downregulated. These functions are closely linked to metabolic processes, particularly redox reactions, substance transport, and the binding of enzyme prosthetic groups.

KEGG enrichment analysis (Figure 7B) identified several significantly downregulated pathways, including tyrosine metabolism, protein processing in the endoplasmic reticulum (involving glycosylation and phosphorylation modifications), protein export, and peroxisome (associated with cellular detoxification). These metabolic activities and protein functions are essential for normal cellular homeostasis. The sustained downregulation of these genes directly impaired the normal growth of *R. solani*, consistent with the inhibition of mycelial growth observed in the earlier assays.

3.6. Metabolomic Results and Analysis

3.6.1. Metabolite Classification and Data Quality Control

Untargeted LC-MS/MS-based metabolomics was employed to characterize the metabolic profiles of mycelial samples from the methyleugenol-treated and control groups.

Correlation analysis of the quality control (QC) samples (Figure 8) revealed that the coefficients of determination (R^2 , the square of the Pearson correlation coefficient) among QC samples approached 1 in both positive and negative ion modes, indicating high analytical stability throughout the detection process and reliable data quality.

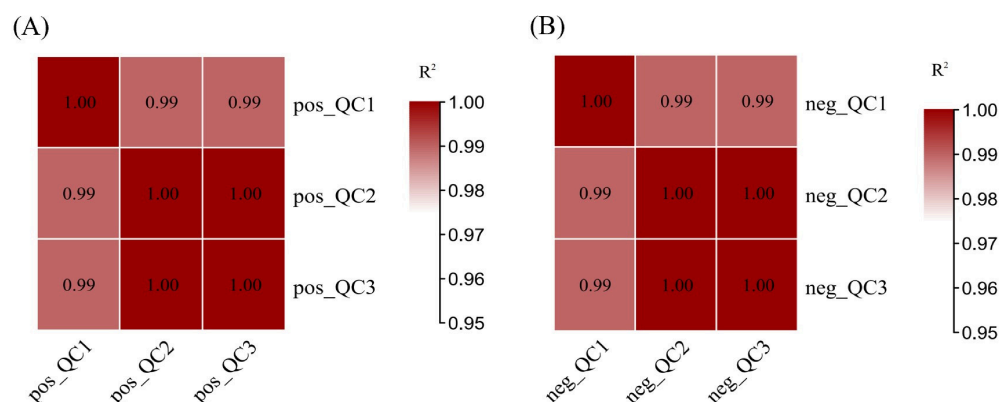


Figure 8. Correlation analysis of the quality control (QC) samples in positive (A) and negative (B) ion modes. Note: The value in each cell represents the coefficient of determination (R^2) between two QC samples; values approaching 1 indicate high analytical stability and reliable data quality.

Metabolite classification statistics (Figure 9) showed that the detected metabolites were predominantly distributed among the following categories: lipids and lipid-like molecules, organic acids and derivatives, nucleosides, nucleotides and analogues, organic oxygen compounds, organoheterocyclic compounds, benzenoids, phenylpropanoids and polyketides, and organic nitrogen compound.

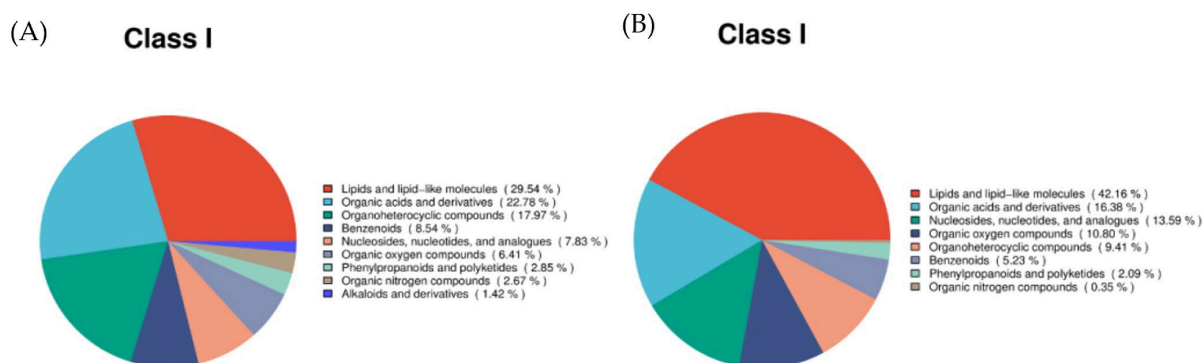


Figure 9. Pie chart of metabolite classification. (A) positive ion mode; (B) Negative ion mode. Note: In both panels, “Class I” refers to the same first-level classification of metabolites; the two pie charts present the proportion of each category detected under the respective ionization mode.

Principal component analysis (PCA) of all samples (Figure 10) demonstrated substantial differences among the treatment groups, with good intra-group reproducibility. The QC samples clustered tightly, further confirming the stability and reliability of the analytical method.

3.6.2. Principal Component Analysis and Screening of Differential Metabolites

PCA results (Figure 11) revealed clear separation between the treated and control groups at both time points in the positive and negative ion modes; specifically, the 4-day treatment group (B) was distinctly separated from the 4-day control group (A), and the 6-day treatment group (D) from the 6-day control group (C). In contrast, no marked

difference was observed between the two control time points (A and C), consistent with the transcriptomic PCA results.

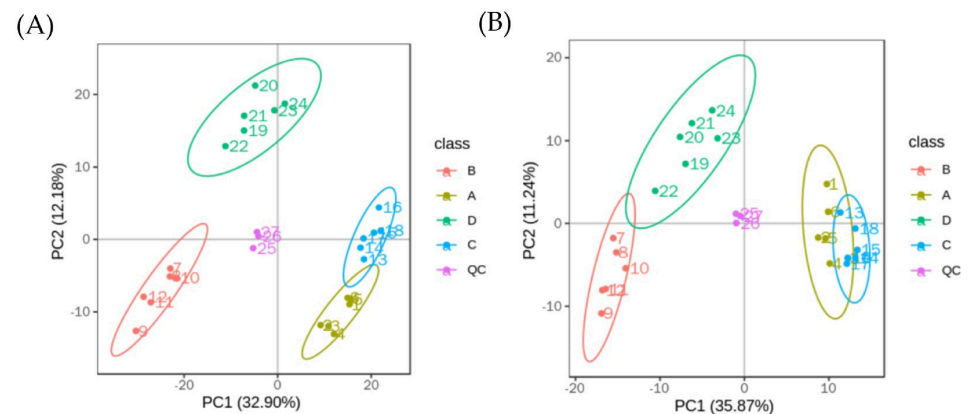


Figure 10. PCA of total samples. (A) positive ion mode; (B) Negative ion mode.

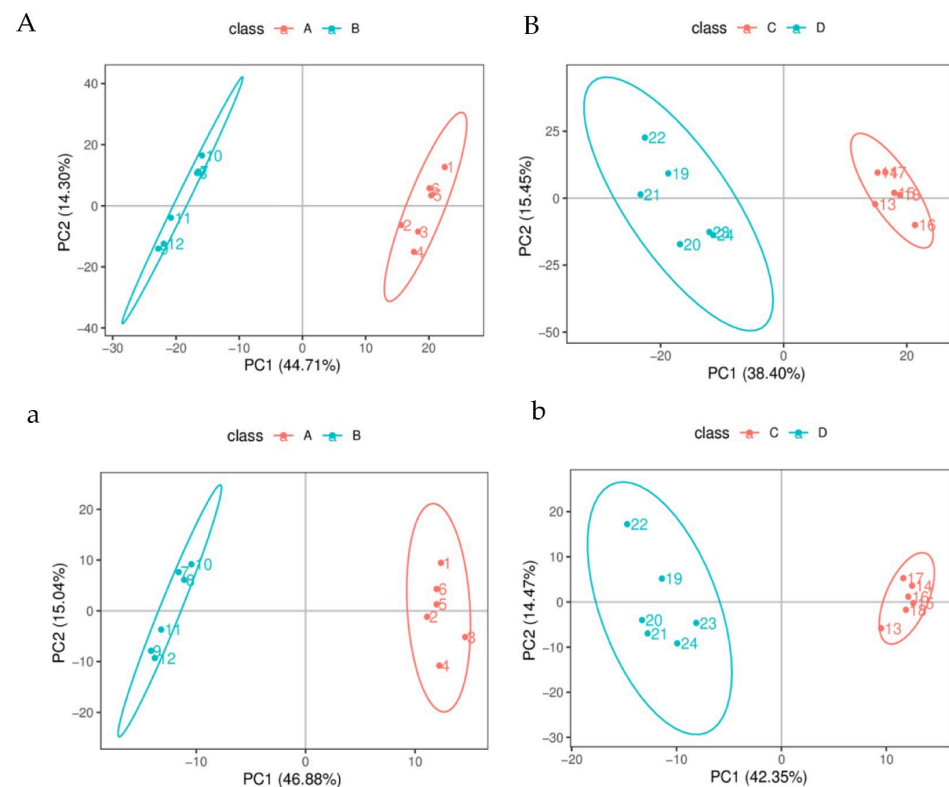


Figure 11. Principal component analysis (PCA). (A,B) positive mode; (a,b) negative ion mode. Note: The abscissa PC1 and ordinate PC2 in the figure represent the scores of the first and second principal components, respectively, and the scatters of different colors represent the samples of different experimental groups, with an ellipse of 95% confidence interval.

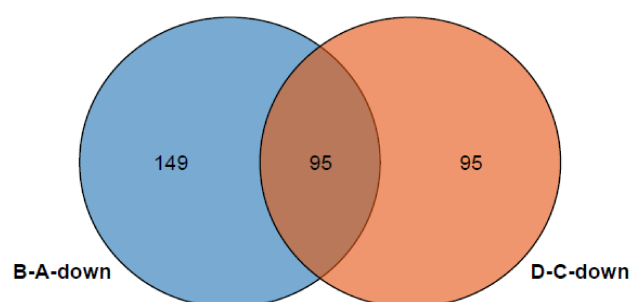
The screening of differential metabolites (Table 5) yielded the following findings. In the positive ion mode, 353 significantly differential metabolites were identified in the B/A comparison (185 upregulated and 168 downregulated), and 274 in the D/C comparison (144 upregulated and 130 downregulated). In the negative ion mode, 139 significantly differential metabolites were identified in the B/A comparison (63 upregulated and 76 downregulated), and 123 in the D/C comparison (58 upregulated and 65 downregulated).

Table 5. Screening results of metabolite differences.

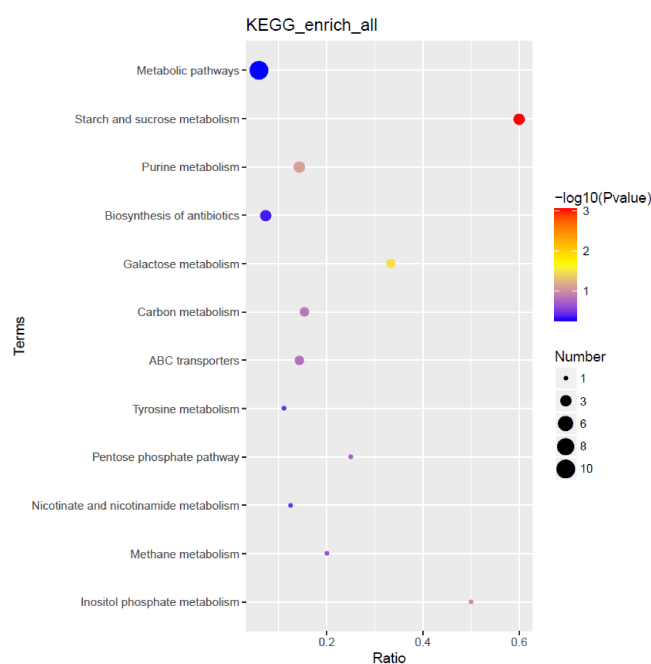
Compared Samples	Num. of Total Ident.	Num. of Total Sig.	Num. of Sig. Up	Num. of Sig. Down
B.vs.A_pos	905	353	185	168
D.vs.C_pos	905	274	144	130
B.vs.A_neg	360	139	63	76
D.vs.C_neg	360	123	58	65

3.6.3. Analysis of Commonly Downregulated Differential Metabolites

Venn diagram analysis (Figure 12) identified 95 metabolites that were consistently down-regulated in the treatment groups relative to their respective controls at both 4 and 6 days.

**Figure 12.** Common and unique metabolites downregulated compared to the CK group in the treatment of 4 d and 6 d.

KEGG pathway enrichment analysis of these 95 commonly downregulated metabolites (Figure 13) revealed that they were primarily enriched in the following pathways: starch and sucrose metabolism, galactose metabolism, purine metabolism, inositol phosphate metabolism, carbon metabolism, ABC transporters, the pentose phosphate pathway, methane metabolism, nicotinate and nicotinamide metabolism, tyrosine metabolism, and biosynthesis of antibiotics.

**Figure 13.** KEGG enrichment analysis of common metabolites down-regulated by methyl eugenol treatment for 4 d and 6 d compared with the CK group.

Among these, the starch and sucrose metabolism and galactose metabolism pathways were significantly enriched ($p < 0.05$), indicating that methyleugenol markedly impaired the carbon-source utilization capacity of *Rhizoctonia solani*. This impairment likely prevented the efficient utilization of carbohydrates such as starch, sucrose, and galactose, and the consequent deficiency in energy conversion may have compromised the maintenance of normal cellular activities. Furthermore, the significant suppression of the antibiotic biosynthesis pathway may have reduced the competitive fitness of *R. solani* within the soil microbial community.

3.7. Association Analysis of Differentially Expressed Genes and Differentially Accumulated Metabolites

Pearson correlation analysis was performed between the 836 genes commonly down-regulated in both the B/A and D/C comparisons and the 95 commonly downregulated metabolites, and a correlation network was constructed (Figure 14).

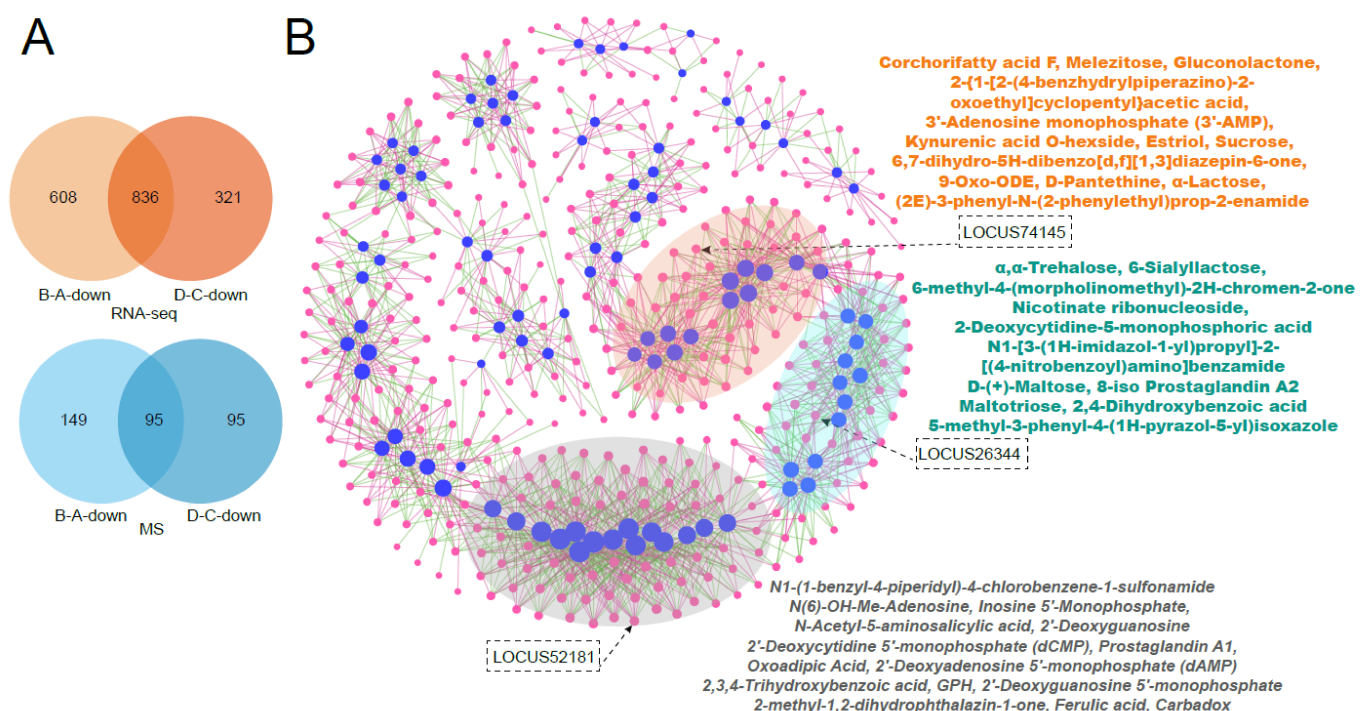


Figure 14. Correlation network diagram. (A) Venn diagram of differentially expressed genes, (B) Network diagram of transcription and metabolic correlation analysis. Note: The boxes in the figure represent metabolites and genes, the color of the box distinguishes metabolites and genes, the yellow box represents metabolites, and the blue box represents metabolites, and the orange box represents genes; The color of the line indicates the correlation, the positive correlation is pink, the negative correlation is green, and the greater the correlation coefficient, the darker the color. In figure B, the three text colors and their corresponding shaded areas indicate the metabolites putatively regulated by each of the three key genes: orange represents metabolites associated with *gene-RDB_LOCUS74145*, green represents those associated with *gene-RDB_LOCUS26344*, and gray represents those associated with *gene-RDB_LOCUS52181*.

This integrated analysis identified three key transcription factors involved in the regulation of multiple metabolites:

- (1) *gene-RDB_LOCUS74145*, which was associated with the production of adenosine 3'-monophosphate, kynurenic acid, estriol, corchorifatty acid F, melezitose, glucono-1,5-lactone, sucrose, and α-lactose, primarily implicating its role in carbohydrate metabolism.

- (2) *gene-RDB_LOCUS26344*, which was associated with the production of α,α -trehalose, nicotinamide riboside, 5'-deoxycytidine monophosphate, maltotriose, and 2,4-dihydroxybenzoic acid, also predominantly involved in carbohydrate metabolism.
- (3) *gene-RDB_LOCUS52181*, which was associated with the production of inosine monophosphate, 5-acetylaminosalicylic acid, deoxycytidine 5'-monophosphate, 2'-deoxyadenosine-5'-monophosphate, 2,3,4-trihydroxybenzoic acid, disodium 2'-deoxyguanosine-5'-phosphate, and ferulic acid, more closely linked to the regulation of the tricarboxylic acid (TCA) cycle and nucleotide metabolism.

These findings suggest that methyleugenol may suppress the expression of these key transcription factors, thereby disrupting carbon-source uptake and utilization as well as energy metabolism via the TCA cycle. Through this dual action on carbon supply and energy production, methyleugenol may synergistically inhibit the growth and development of *R. solani*.

4. Discussion

4.1. Potential of Methyleugenol as a Plant-Derived Fungicide

In this study, we systematically evaluated, for the first time, the antifungal activity of methyleugenol—the principal component of *Asarum* essential oil—against *Rhizoctonia solani* in ginseng. Methyleugenol markedly inhibited *R. solani*, with an inhibition rate of 51% after 4 days of treatment at 0.1 mg/mL and an EC₅₀ of 60 mg/L. The antifungal potency of methyleugenol observed here (EC₅₀ = 60 mg/L) is comparable to, and in several cases exceeds, that of other plant-derived compounds reported against *Rhizoctonia* spp. [32]. As the major constituent of *Asarum* essential oil, methyleugenol is widely available, structurally well-defined, and readily obtainable. It has also been shown to control soil nematodes, which exhibited a 24 h median lethal concentration (LC₅₀) of 907 mg/L against *Caenorhabditis elegans* [21], suggesting that this multifunctional, plant-derived soil amendment holds considerable promise for the integrated management of soil-borne diseases in ginseng.

With prolonged incubation, the inhibition rate of methyleugenol first plateaued and then declined. This trend may be attributed to the gradual volatilization and loss of the compound in the liquid culture system [32], or to the metabolic adaptation of *R. solani* to sublethal concentrations. These observations indicate that, in practical applications, slow-release formulations or repeated applications may be required to sustain efficacy.

Purified methyleugenol offers a chemically defined, reproducible active ingredient with predictable dose–response behaviour, whereas the crude *Asarum* essential oil—of which methyleugenol is the principal constituent—may be more economical and could provide additional activity through minor co-constituents, albeit at the cost of batch-to-batch variability. Our current data support that the essential oil may represent a lower-cost alternative for large-scale soil application.

Because methyleugenol is poorly soluble in water, it is difficult to formulate as an aqueous solution for irrigation or foliar spraying. Instead, a more practical strategy under field conditions may be to intercrop *Asarum* with ginseng. Owing to the volatile and readily diffusible nature of methyleugenol, the compound released from the intercropped *Asarum* plants is expected to exert an effective inhibitory action on *R. solani* within a certain range, thereby suppressing damping-off disease in the field. The spatial extent over which such protection can be achieved remains to be determined; accordingly, we plan to evaluate different intercropping layouts—for example, one, two, three, or five rows of *Asarum*—to define the effective range of biocontrol. Notably, *Asarum* and ginseng are both shade-loving plants with similar cultivation and field-management requirements, and both are high-value medicinal crops. Intercropping the two species may therefore suppress soil-borne

disease while simultaneously generating additional economic returns. Most importantly, this approach could reduce the input of synthetic fungicides and thereby mitigate their contamination of soil and the surrounding environment, offering an environmentally friendly avenue for the green control of ginseng damping-off disease.

4.2. Pronounced Inhibition of Carbon Metabolism

Integrated transcriptomic and metabolomic analyses revealed that methyleugenol substantially suppressed carbon metabolism in *R. solani*. KEGG enrichment analysis of the metabolome identified starch and sucrose metabolism and galactose metabolism as the most significantly down-regulated pathways. Carbon metabolism is the fundamental route by which fungi acquire energy and carbon skeletons [33]. As a typical saprophytic, soil-borne pathogen, *R. solani* obtains nutrients mainly by secreting cell wall-degrading enzymes that break down host cell wall polysaccharides [34]. By suppressing carbon metabolism, methyleugenol may prevent the pathogen from efficiently utilizing environmental carbohydrates, thereby restricting hyphal growth and infection.

Similar mechanisms have been reported for other antifungal compounds. Li et al. [35] showed that cinnamaldehyde inhibits *Aspergillus flavus* by suppressing carbohydrate metabolism. In the present study, the simultaneous inhibition of starch, sucrose, and galactose metabolism by methyleugenol may produce a more effective metabolic blockade, which could partly account for its high antifungal efficiency.

4.3. Sustained Disruption of Protein Processing and Export

Transcriptomic analysis showed that protein processing in the endoplasmic reticulum (ER) and protein export were consistently enriched among the down-regulated pathways at both 4 and 6 days of treatment. The ER is the central organelle for protein folding, glycosylation, and quality control in fungal cells [36], whereas the protein export pathway mediates the secretion of proteins, including virulence factors such as cell wall-degrading enzymes and toxins [37].

Inhibition of these pathways by methyleugenol may have several consequences: (1) impaired folding of newly synthesized proteins, triggering the unfolded protein response (UPR) and ER stress [38,39]; (2) blocked synthesis and secretion of pathogenic factors such as cell wall-degrading enzymes, thereby weakening virulence [40]; and (3) disrupted assembly of membrane proteins and transporters, which compromises transmembrane nutrient transport. Together, these effects may act synergistically to cause cellular dysfunction and growth inhibition.

4.4. Disruption of Redox Homeostasis and Peroxisomal Function

GO analysis indicated that, among the 836 commonly down-regulated genes, functions such as oxidoreductase activity, iron ion binding, and heme binding were significantly enriched in the down-regulated set. Consistently, KEGG analysis showed marked suppression of the peroxisome pathway. Peroxisomes are key sites of reactive oxygen species (ROS) metabolism and fatty acid β -oxidation in fungal cells [41]. Suppression of this pathway may lead to: (1) a reduced capacity to scavenge intracellular ROS, resulting in oxidative damage [42]; (2) impaired fatty acid metabolism, affecting membrane integrity and energy supply; and (3) decreased tolerance to the host's defensive ROS burst, thereby weakening the pathogen's fitness during host–pathogen interactions [43].

This finding is consistent with the mechanism reported by Zhao et al. [13], in which antifungal compounds act by disrupting the redox balance of pathogens. In the present study, methyleugenol may exert its antifungal effect, in part, by interfering with ROS metabolism and redox homeostasis in *R. solani*.

4.5. A Multilevel Regulatory Network Mediated by Key Transcription Factors

Integrated transcriptomic and metabolomic analysis identified three key transcription factors (gene-RDB_LOCUS74145, gene-RDB_LOCUS26344, and gene-RDB_LOCUS52181) that were persistently down-regulated following methyleugenol treatment, each regulating the production of multiple metabolites. Gene-RDB_LOCUS74145 and gene-RDB_LOCUS26344 were primarily associated with carbohydrate-related metabolites (e.g., sucrose, α -lactose, melezitose, trehalose, and maltotriose); their down-regulation may directly suppress carbon uptake and utilization. In contrast, gene-RDB_LOCUS52181 was more closely linked to nucleotide and organic acid metabolites, which are central to energy metabolism via the tricarboxylic acid (TCA) cycle.

In fungi, transcription factors that regulate carbon metabolism—such as Mig1 and Snf1 in *Saccharomyces cerevisiae*—control the expression of carbon utilization genes in response to glucose signaling [44–46]. The three transcription factors identified here may serve analogous roles. Their suppression by methyleugenol could cascade through downstream carbon and energy metabolism gene networks, producing a multilevel effect characterized by impaired carbon utilization, insufficient energy supply, and inhibited growth and development. Nevertheless, the specific downstream genes regulated by these factors, and the signaling pathways through which they sense methyleugenol, remain to be determined through functional validation, including gene knockout and overexpression experiments.

4.6. An Integrated Model of the Antifungal Mechanism of Methyleugenol

Based on the integrated transcriptomic and metabolomic results, we propose a multi-target, synergistic model for the inhibition of *R. solani* by methyleugenol (Figure 14). Methyleugenol may first act on the cell membrane or enter the cell via passive diffusion [47], and subsequently inhibit pathogen growth through several coordinated routes: (1) suppressing the transcription of genes encoding key enzymes in carbon metabolism, thereby blocking the utilization of carbohydrates such as starch, sucrose, and galactose and cutting off the nutrient supply; (2) interfering with protein folding and processing in the ER, thereby impairing the synthesis and secretion of functional proteins; (3) disrupting redox homeostasis and peroxisomal function, thereby inducing oxidative stress; and (4) suppressing the expression of key transcription factors, which cascades to the TCA cycle and nucleotide metabolism and results in an inadequate energy supply. Together, these multi-target effects ultimately lead to the marked inhibition of *R. solani* hyphal growth.

4.7. Future Perspectives

Our findings open several avenues that we intend to pursue, following a logical progression from deepening the mechanistic understanding to translating it into practical application. Mechanistically, our foremost priority is to establish the regulatory roles of the three key transcription factors uncovered by our integrated transcriptome–metabolome analysis. We hypothesize that these factors act as central hubs coordinating carbohydrate metabolism and the tricarboxylic acid cycle in *R. solani*, and we plan to test this hypothesis directly by combining CRISPR-Cas9 gene editing with DNA-binding and protein–protein interaction assays to define their target genes and downstream networks. In parallel, we may aim to resolve how methyleugenol enters fungal cells and to identify its primary molecular targets; to this end, we will employ molecular docking and affinity chromatography to capture its direct binding partners and thereby clarify the basis of its multi-target mode of action.

We further anticipate that pairing methyleugenol with biocontrol agents or low-toxicity chemical partners will yield synergistic effects that support an integrated management strategy. Ultimately, we will validate these strategies through systematic field trials, while carefully

assessing their impact on non-target soil microbial communities, so as to fully establish the practical potential and ecological safety of methyleugenol—whether applied as a slow-release formulation or delivered through Asarum intercropping—as a botanical approach for the green control of ginseng damping-off disease, with the added benefit of reducing synthetic fungicide inputs and their contamination of soil and the surrounding environment.

5. Conclusions

In this study, we set out to identify a plant-derived antifungal agent from Asarum heterotropoides essential oil and to clarify its mode of action against *Rhizoctonia solani*, the causal agent of ginseng damping-off disease.

We demonstrated that methyleugenol is the predominant volatile constituent of the essential oil and possesses pronounced, concentration-dependent antifungal activity against *Rhizoctonia solani*. Through integrated transcriptomic and metabolomic profiling, we established that methyleugenol suppresses the pathogen through a coordinated, multi-target mode of action rather than a single pathway.

We further identified three key transcription factors as central regulatory candidates underlying this response. Collectively, these results answer our initial objectives by confirming methyleugenol as a promising botanical fungicide and by defining its principal molecular targets, thereby providing a foundation for green strategies to manage ginseng damping-off disease.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agriculture16141547/s1>, Figure S1. Colony morphology of *Rhizoctonia solani* after treatment with different concentrations of methyl eugenol for 4 days. Note: A: CK; B: 0.1 mg/mL; C: 0.05 mg/mL; D: 0.01 mg/mL. Figure S2. Colony morphology of *Rhizoctonia solani* after treatment with different concentrations of methyl eugenol for 6 days. Note: A: CK; B: 0.1 mg/mL; C: 0.05 mg/mL; D: 0.01 mg/mL. Figure S3. Colony morphology of *Rhizoctonia solani* after treatment with different concentrations of methyl eugenol for 8 days. Note: A: CK; B: 0.1 mg/mL; C: 0.05 mg/mL; D: 0.01 mg/mL.

Author Contributions: Conceptualization, R.Z., B.L. and Y.W.; methodology, R.Z., X.C. and B.L.; software, R.Z. and X.Y.; validation, R.Z., X.Y., B.L. and Y.W.; formal analysis, R.Z. and B.L.; investigation, R.Z., Z.Y. and D.Z.; resources, Y.W.; data curation, R.Z., X.Y. and B.L.; writing—original draft preparation, R.Z.; writing—review and editing, B.L., X.C. and Y.W.; visualization, R.Z.; supervision, B.L. and Y.W.; project administration, B.L. and Y.W.; funding acquisition, R.Z. and Y.W. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

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