

To investigate the antimicrobial active substances of the endophytic fungus *Achaetomium sophora* HY17 from *Sophora alopecuroides* in the co-culture system based on UPLC-MS/MS technology

Guanlan Liu

Zhiyun Tang

Routing Wang

Peiwen Gu

gupeiwen2019@nxu.edu.cn

Research Article

Keywords: Endophytic fungi, *Achaetomium*, *Botrytis cinerea*, Co-culture, Metabolomics

Posted Date: August 13th, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-7129029/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Abstract

The secondary metabolites produced by endophytic fungi of medicinal plants have a broad potential in the field of biological control. Metabolomics and microbial co-culture technology are the common and crucial methods for exploring microbial secondary metabolites. The endophytic fungus *A. sophora* HY17, isolated from the medicinal plant *Sophora alopecuroides*, was used in this study to investigate the dynamic changes in secondary metabolites during its interaction with the pathogenic fungi *B. cinerea* HM. And the metabolites were quantified with Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) and metabolomics. The results show that *A. sophora* HY17 contained various metabolites such as alkaloids, flavonoids, phenolic acids, terpenoids, lignans, and coumarin. Further, through the established metabolomics model, sophoridine, sophocarpine, matrine, oleoylethanolamine, 3-hydroxyanthranilic acid, chalcone, cynaroside, daidzein, vitexin, and 1-tetralone were identified to play significant antifungal roles in the interaction between the two fungi. The KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis revealed a significant enrichment of differential secondary metabolites in the phenylalanine metabolic pathway throughout the interaction. This study provides a theoretical basis for identifying antifungal secondary metabolites produced by plant endophytic fungi and designing new biocontrol agents.

Introduction

Microbial metabolites are valuable resources for discovering new antibacterial compounds (Dinglasan et al. 2025) in biological pest control investigation. Endophytic fungi from medicinal plants have gained attention as novel microbial sources in recent years. The secondary metabolites of endophytic fungi in most medicinal plants contain bioactive substances that can inhibit pathogenic fungi, making them potential valuable materials for discovering new antifungal agents. Isabel et al. (2023) demonstrated the positive impact of *Trichoderma* sp., an endophytic fungus, by isolating a novel polyketide compound trichoketide F. This compound exhibited a potent inhibitory activity against *Sclerotinia sclerotiorum* at a minimum inhibitory concentration (MIC) of 12.5 µg/mL. Chen et al. (2019) discovered new sesquiterpenoids leptosphin A and B in the solid fermentation culture of *Leptosphaeria* sp. XL026, which are endophytic fungus living in the leaves of the medicinal plant *Panax notoginseng*. Leptosphin B exhibited significant inhibitory activity against *Bacillus subtilis*. Zhu et al. (2017) isolated penochalasin K, a novel hexacyclic skeleton, from the metabolite of mangrove endophytic fungus *Penicillium chrysogenum* V11. It exhibited significant inhibitory activity against *Colletotrichum gloeosporioides* and *Rhizoctonia solani*, with MIC values of 6.13 and 12.26 µM, respectively. Such inhibition effect surpasses that of carbendazim. Therefore, the active metabolites synthesis by the endophytic fungi in medicinal plants provided a significant resource for identifying new compounds with antifungal activities, thus screening these secondary metabolites for effective antifungal compounds is of significance (Gokhale et al. 2017).

In recent years, fungal-plant co-culture has been commonly accepted as a promising approach for discovering novel antimicrobial metabolites (Ahmad et al. 2020). When cultured individually, Studies

have revealed that most microorganisms' gene clusters remain inactive. On the contrary, the co-culture strategy can activate these silent genes by altering external conditions with necessary cofactors. This activation promotes the production of numerous secondary metabolites with unique structures and significant physiological activities (Bode et al. 2002). To some extent, microbial co-culture simulates the complex living conditions of microorganisms in their natural environment (Romano et al. 2018). This simulation utilizes interactions such as survival competition between multiple microorganisms to stimulate the production of metabolites, both in their diversities and quantities (Partida-Martinez L P, Hertweck C 2005). The differential metabolites in co-culture are closely associated with microorganisms' chemical defense mechanisms and often possess various biological activities, such as antibacterial and cytotoxic effects (Whitt et al. 2014). There are multiple successful examples of using the microbial co-culture approach in exploring the potential synthesis of microbial secondary metabolites (Peng et al. 2021). Chagas et al. (2013) co-cultured two endophytic fungi, *Alternaria venezissima* and *Nigrospora sphaerica*, and isolated two new polyketide compounds: stemphyperlenol and alterperlenol. Stemphyperlenol inhibits *N. sphaerica*. Liu et al. (2022) discovered that when *Aspergillus oryzae* and *Zygosaccharomyces rouxii* were co-cultured, *A. oryzae* produced Taurine, Imidazoleacetic acid, and other substances. These substances significantly inhibited the growth of *Z. rouxii*, indicating that fungal co-culture can impact the yield and types of secondary metabolites.

The *Achaetomium* fungus is abundant and can be found in various decaying plant residues, fibrous materials like paper and wood, seeds, leather, animal excreta, and different plant tissues (Wang et al. 2016). It has wide applications in the food industry, husbandry, and other agriculture fields. So far, studies have found that the metabolites of hairless trichothecium fungi (belong to *Achaetomium* fungus) mainly contain murewall degrading enzymes like mannanase (Zhao et al. 2013) and poly-galacturonase (Tu et al. 2013), where mannanase is a crucial hemicellulase and widely utilized in husbandry, paper making, detergent production, food processing, and oil extraction. Polygalacturonase functions as a pectinase and finds extensive applications in fruit juice production, paper making, textile industry, and oil extraction. Therefore, the *Achaetomium* fungus has gained increasing attention in industrial production and scientific research; however, limited studies have been conducted on its biological control.

Achaetomium sp. HY17, an endophytic fungus isolated from *Sophora alopecuroides* cotyledon (Wang et al. 2022), has been found to possess growth-promoting activities such as phosphorus solubilization, gibberellin production, siderophore secretion, cellulase synthesis, and protease production. Moreover, *Achaetomium* sp. HY17 exhibited significant effects against several pathogenic fungi, including *Botrytis cinerea* HM1, *Rhizoctonia solani* pn5-2, *Fusarium avenacea* YM1, *Fusarium oxysporum* pm29-3, *Colletotrichum siamense* NX2-7, *Fusarium tricuspidata* pm36-8, *Clonostachys rosea* pm34-5 and *Pythium aphanidermatum* pn8-3, making it a promising candidate for biocontrol development. However, the production mechanism and action of its antimicrobial substances remain unclear. The study investigated the dynamic changes in secondary metabolites of *Achaetomium* sp. HY17 during its interaction with *B. cinerea* HM1 using co-culture and metabolomics technology, leading to the discovery of antibacterial secondary metabolites. This research provides a theoretical foundation for designing and developing biocontrol agents.

1 Materials and methods

1.1 Test strain and standard substance

The biocontrol fungus *Achaetomium* sp. HY17 strain (CGMCC No.41060) was deposited at the General Microbiology Center of the Chinese Committee for Microbial Culture Preservation.

The pathogenic fungi, including *Botrytis cinerea* HM1, *Rhizoctonia solani* pn5-2, *Fusarium avenacea* YM1, *Fusarium oxysporum* pm29-3, *Colletotrichum siamense* NX2-7, *Fusarium tricuspidata* pm36-8, *Clonostachys rosea* pm34-5 and *Pythium aphanidermatum* pn8-3 were isolated and preserved by the Plant Pathology Laboratory, College of Agriculture, Ningxia University.

The test standard products, including sophoridine (HPLC $\geq$ 98%), sophocarpine (HPLC $\geq$ 98%), matrine (HPLC $\geq$ 98%), oleoylethanolamine (HPLC $\geq$ 90%), 3-hydroxyanthranilic acid (10 mM in DMSO), chalcone (HPLC $\geq$ 98%), cynaroside (HPLC $\geq$ 98%), daidzein (HPLC $\geq$ 95%), vitexin (HPLC $\geq$ 98.5%) and 1-tetralone (HPLC $\geq$ 98%) were purchased from Shanghai Yuanye Biotechnology Co., LTD.

1.2 Evaluation of antifungal activity

The in-dish confrontation method (An et al. 2020) was used to inoculate *Achaetomium* sp. HY17 and *B. cinerea* HM1 mycelial disks (5 mm in diameter) on PDA medium, with the two mycelial disks placed 4 cm apart. Two experimental treatments were conducted: simultaneous inoculation of biocontrol and pathogenic fungi, and delivery of fungal control for 3 days prior to pathogenic fungi inoculation. Three replicates were set up for each treatment. The dish were incubated at 25 °C with only pathogenic fungi as the blank control group. When the pathogenic fungi in the blank control group almost filled the petri dish, measurements were taken for both the colony radius (R_c) and the extension of colony growth towards antifungal control (R) in the experimental group, from which inhibition rate was calculated.

$$\text{inhibition rate (\%)} = [(R_c - R)/(R_c - 2.5)] \times 100$$

1.3 Classification status identification

According to the classical classification method of fungi, morphological identification was conducted based on strain colony characteristics such as shape, texture, color (Mehrabi et al. 2020). For molecular identification, fungal genomic DNA extraction kit was used for DNA extraction. Primers ITS1/ITS4 (White et al. 1990), LROR/LR3 (Rehner et al. 1995), and Bt2a/Bt2b (Glass et al. 1995) were designed for PCR amplification reactions targeting internal transcription spacer 1 and 2 of fungal 5.8S nrDNA, D1/D2 domain of 28S nrDNA, and β -tubulin gene sequence respectively. The PCR reaction system (25 μ L) consisted of master mix (12.5 μ L), DNA (2 μ L), forward and reverse primers (1 μ L each), supplemented with ddH₂O. The PCR reaction conditions included 34 cycles of predenaturation at 94 °C for 3 min, followed by denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 10 min. The PCR products were detected using a spectrophotometer and analyzed through agarose gel electrophoresis (1%). Subsequently, the samples

were sent to Sangon Bioengineering (Shanghai) Co., LTD for sequencing. Sequence information with high homology was searched and downloaded from NCBI BLAST database. MEGA7 software and the Neighbour-Joining method were employed to construct the phylogenetic tree (Wang et al. 2019).

1.4 Co-culture sample collection

Refer to Triastuti et al.'s method (2021). The fungi *A. sophora* HY17 and *B. cinerea* HM1 were co-cultured on PDA medium, with their mycelial disks placed 4 cm apart and incubated at 25 °C. At 3 dpi, 6 dpi, and 10 dpi when the mycelium of both fungi made contact, a sterilized slide was used to scrape off 1 g of mycelium from the co-culture (CO) area, which was then transferred into a 5 mL frozen storage tube as one sample. This process was performed in triplicate. Blank controls were prepared by culturing colonies of *A. sophora* HY17 (Ch) and *B. cinerea* HM1 (HM) separately under identical culture conditions. The samples were labeled as Ch-3d (*A. sophora* HY17 mono-culture for 3 days), HM-3d (*B. cinerea* HM1 mono-culture for 3 days), CO-3d (*A. sophora* HY17 and *B. cinerea* HM1 co-culture for 3 days), Ch-6d (*A. sophora* HY17 mono-culture for 6 days), HM-6d (*B. cinerea* HM1 mono-culture for 6 days), CO-6d (*A. sophora* HY17 and *B. cinerea* HM1 co-culture for 6 days), Ch-10d (*A. sophora* HY17 mono-culture for 10 days), HM-10d (*B. cinerea* HM1 mono-culture for 10 days), and CO-10d (*A. sophora* HY17 and *B. cinerea* HM1 co-culture for 10 days). All samples were snap-frozen in liquid nitrogen for a duration of 5 minutes and stored at -80 °C until use.

1.5 UPLC-MS/MS detection and analysis

1.5.1 Sample extraction

1.4 samples were thawed, vortexed for 1 min, mixed well, and then added with a 20 °C solution of 70% methanol water internal standard extract at a ratio of 600 μL per 50 mg sample. After vortexing for 15 min, the samples were centrifuged at $12000\text{ rpm}\cdot\text{min}^{-1}$ and 4 °C for 3 min. The supernatant was filtered through a microporous filter membrane with a pore size of 0.22 μm and stored in an injection bottle for subsequent LC-MS/MS detection.

1.5.2 UPLC-MS/MS detection

The UPLC system (Exion LC™ AD, <https://sciex.com.cn/>) and MS/MS instrument (Applied Biosystems 6500 QTRAP, <https://sciex.com.cn/>) were used for data acquisition. An Agilent SB-C18 column (100 x 2.1 mm, 1.8 μm) was employed with a flow rate of $0.35\text{ mL}\cdot\text{min}^{-1}$ and a column temperature of 40 °C to inject 2 μL of ultra-pure water (0.1% formic acid) and acetonitrile (0.1% formic acid) as mobile phases. The ESI source temperature was set at 500 °C, while the IS voltage was set at + 5500 V in positive ion mode and - 4500 V in negative ion mode. GSI, GSII, and CUR gas pressures were maintained at 50 psi, 60 psi, and 25 psi respectively; the collision-induced ionization parameter was set to high; MRM scans were performed using nitrogen as the collision gas on medium setting; DP optimization was conducted.

1.5.3 Statistical analysis of data

Based on the MWDB database established by Metville Biotechnology Co., LTD., the material was characterized using secondary spectrum information. Multiple reaction monitoring (MRM) mode of triple quadrupole mass spectrometry was used for quantitative analysis of each secondary metabolite. The metabolite data from different samples were obtained for qualitative and quantitative analysis using mass spectrometry (Analyst1.6.3). The data were normalized, annotated based on retention time, plasma-nucleus ratio, and peak intensity, and further confirmed using standards for most substances. Multidimensional statistics were employed to establish a stable and reliable mathematical model for metabolite analysis. Firstly, Principal Component Analysis (PCA) with unsupervised pattern recognition was conducted to preliminarily understand overall metabolite differences between sample groups and within-group variations among samples. Partial Least Squares-Discriminant Analysis (PLS-DA), a supervised pattern recognition method, was utilized to identify differential metabolites and determine overall differences in metabolites between groups. Orthogonal partial least squares discriminant analysis (OPLS-DA) was employed to eliminate irrelevant differences and identify differential metabolites among the sample groups. The selection criteria for differential variables were a variable weight value (VIP) greater than 1. Evaluation and analysis of the model were based on R^2X , R^2Y , and Q^2 parameters. A higher value close to 1 indicates a more stable and reliable model. Differential metabolites were screened using VIP values (> 1), Student's t-test with $p < 0.05$, and fold change measurements. Metabolite differences were determined by applying Log2 transformation with $VIP > 1$ and $p < 0.05$ thresholds. Metabolites with $\text{Log}_2\text{FC} \geq 2$ or $\text{Log}_2\text{FC} \leq 0.5$ were considered as differential metabolites. The KEGG PATHWAY database provides information on molecular interactions, physiological reactions, biochemical processes, and gene product relationships in manually mapped metabolic pathways. By aligning metabolite information with KEGG compound IDs, we can obtain insights into the involved metabolic pathways and evaluate their impact on biological processes.

1.6 Determination of the inhibitory activity of differential metabolites

Based on the identified differential metabolites, 10 selected metabolites (listed in section 1.1) were purchased as standards to evaluate their antifungal effect. Following the method of Li et al. (2024), the experiment included a drug group and a blank control group. In the drug group, the tested standard substance was dissolved in methanol, while methanol solution was used in the blank control group. The standard substances were prepared with mass concentrations of 50, 100, 150, 200, 250, and 300 mg/mL using methanol and filtered through a 0.22 μm microporous filter membrane before use. In the drug group, different concentrations of a standard solution (10 μL) were added to each dish, followed by the addition of PDA medium (10 mL) to create a drug-containing medium. The final mass concentrations of the drug solution were 50, 100, 150, 200, 250 and 300 $\mu\text{g/mL}$ respectively. In the blank control group, methanol solution (10 μL) was added in the same manner. After complete cooling of the medium, a mycelial disk with a diameter of 5 mm from *B. cinerea* HM1 was inserted into the center of each plate in all groups. The plates were then incubated at 25 °C in darkness. When fungal colonies almost filled the culture plate in the blank control group, cross-method measurements were taken for determining fungal colony diameters in each treatment group and calculating inhibition rates based on average values.

Prism software was utilized for regression equation calculation as well as EC50 and EC90 determination.

1.7 *A. sophora* HY17 Extraction and determination of alkaloids and flavonoids

1.7.1 Extraction of alkaloids and flavonoids from samples

The isolated and purified strain was inoculated in PDA medium. After 7 days of culture at 26°C, 5 mycelial disks with a diameter of 0.8 cm were inoculated in 150 mL PDB medium. After 7 days of shaking culture at 26°C and 175 r/min, the mycelia were collected by filtration with 4 layers of sterile mesh, and the mycelia were dried to constant weight in an oven at 50°C. 1 g of mycelium was weighed. After grinding the mycelium to powder in a mortar, the mycelium was placed in a 50 mL centrifuge tube, 15 mL methanol was added, mixed evenly, sonicated at 45°C for 60 min, centrifuged at 9500 r/min for 10 min, and the supernatant was evaporated and concentrated to 2 mL on a rotary evaporator after a 0.45 µm filter, and stored at 4°C until examined.

1.7.2 Detection of Alkaloids by HPLC

According to the method of Ju et al. (2022), the chromatographic conditions were: Ultimate AQ-C18 (4.6 µm×250 mm×5 mm); The mobile phase was 0.2% phosphoric acid (triethylamine 2.00 mol/L, A) and acetonitrile (B), with elution gradient of 0 ~ 22 min and 96.5% A; 22 ~ 23 min, 96.5% ~ 0%A; 23 ~ 27 min, 100%A, 27 ~ 28 min, 0% ~ 96.5%A; 28 ~ 34 min, 96.5%A, 10 µL of sample, 1.0 mL/min of flow rate, 205 nm of detection wavelength and 30°C of column temperature. Comparing the HPLC chromatogram of the endophytic fungus sample to be detected with the alkaloid standard, when the sample has a peak with the same or similar retention time as that of the standard, it can be determined that the sample to be detected contains the corresponding alkaloid.

1.7.3 Detection of Flavonoids by HPLC

According to the method of Li et al. (2024), the chromatographic conditions were: Ultimate AQ-C18 (4.6 µm×250 mm×5 mm); Mobile phase A is acetonitrile, and mobile phase B is 0.8% phosphoric acid aqueous solution with elution gradient of 0 ~ 5 min and 18% A; 5 ~ 8 min, 24%A; 8 ~ 10 min, 36%A; 10 ~ 20 min, 38%A; 20 ~ 20.01 min, 45%A; 20.01 ~ 25 min, 45%A; 25 ~ 30 min, 65%A; 30 ~ 35 min, 70%A; 35 ~ 36 min, 71%A; 36 ~ 38 min, 18%A; The injection volume is 10 µL, the flow rate is 0.8 mL/min, the detection wavelength is 225nm, and the column temperature is 25°C. Comparing the HPLC chromatogram of the endophytic fungus sample to be detected with the flavonoid standard, when the sample has a peak with the same or similar retention time as that of the standard, it can be determined that the sample to be detected contains the corresponding flavonoid.

2 Results and analysis

2.1 Refinement and categorization of the antifungal potential of endophytic fungus HY17.

The results of fungal antagonism in Petri dishes showed that when fungi HY17 and pathogenic fungi were inoculated simultaneously, fungus HY17 exhibited the strongest antifungal effect on *B. cinerea* HM1, with an inhibition rate of 74.60%. The inhibition rate of *C. siamense* NX2-7 was 68.08%. When fungus HY17 was inoculated 3 days prior to pathogenic fungi, it significantly enhanced the inhibitory effect on seven strains of pathogenic fungi: *B. cinerea* HM1, *R. solani* pn5-2, *F. avenacea* YM1, *F. oxysporum* pm29-3, *C. siamense* NX2-7, *F. tricuspidata* pm36-8 and *P. aphanidermatum* pn8-3. Among them, the inhibition rate for *B. cinerea* HM1, *R. solani* pn5-2, *F. avenacea* YM1 and *C. siamense* NX2-7 increased to over 90% (Table S1).

By observing fungus HY17 on PDA medium, it was found that the colony had a light pink and cotton-like appearance (Fig. 1A). The aerial mycelium initially appeared white but later turned light pink, while the back surface of the culture became reddish-brown (Fig. 1B). After 25 days of incubation at 28°C, the ascus shell matured. It exhibited a dark brown or black color and could be either supergenic or buried in aerial hyphae. The ascus shell had pores and a spherical shape, with scattered, uneven, and irregularly branching accessory filaments covering its surface (Fig. 1C). The asci were clubbed and transparent, containing oval-shaped ascospores (Fig. 1D-ab). Additionally, there were chlamydospores present with a four-horned star shape (Fig. 1D-cd). These morphological characteristics bear some resemblance to those of the revised *Achaetomium* fungus. Based on morphological identification, a phylogenetic tree was constructed using the tandem sequences of *ITS-LSU-TUB* multigenes (Fig. 1E). The tree revealed that fungus HY17 is closely related to *Achaetomium* sp., with fungi clustering together with 100% support. However, fungus HY17 was genetically distant from other fungi whose species names have been determined, such as *Achaetomium cristalliferum* (CBS 781.84), *Achaetomium strumarium* (CBS 333.67), *Achaetomium aegilopis* (IRAN 3453C) and *Achaetomium globosum* (CBS 119.76) and formed an independent branch. Combining the morphological characteristics of fungal HY17 and the alignment results based on the *ITS-LSU-TUB* multi-gene tandem sequence (Fig. 1), fungus HY17 was identified as a new species belonging to Ascomycotina, Pyrenomycetes, Sordariales, Chaetomiaceae, and *Achaetomium*. Since it was first isolated from *sophora alopecuroides*, it has been named *Achaetomium sophora* HY17. The *A. sophora* HY17 preservation number is CGMCC No.41060, and it has been deposited in the General Microbiology Center of the Chinese Committee for Microbial Culture Preservation.

2.2 Morphological alterations of co-cultivated fungal mycelia

The mycelia morphology of *A. sophora* HY17 and *B. cinerea* HM1 changed significantly during 0–10 days of co-culture compared to when they were cultured alone. Contact between *A. sophora* HY17 and *B. cinerea* HM1 was observed at 3 dpi, followed by obvious inhibition at 4 dpi with a light pink inhibition

zone in the contact area. Over time, the color of *A. sophora* HY17 colony and the contact area between the two fungi deepened significantly (Fig. 2).

2.3 Quality control of metabolomics samples and comprehensive sample analysis

The instrument's high stability guarantees the repeatability and reliability of the data. UPLC-MS/MS was used to analyze three replicates of individual cultures (Ch/HM) and co-cultures (CO) of *A. sophora* HY17 and *B. cinerea* HM1 at three time points (3 dpi, 6 dpi, and 10 dpi). Overlapping display analysis of total ion current maps (TIC graphs) for different quality control QC samples showed that the metabolite detection in negative ionization mode (NI) and positive ionization mode (PI) had consistent retention time and peak intensity. These results indicate good signal stability of mass spectrometry when detecting the same sample at different times. The Pearson's Correlation Coefficient r was used to evaluate the correlation of biological replicates. The sample's $|r|$ is close to 1, indicating strong correlation and high data reliability. Principal Component Analysis (PCA), a non-supervisory statistical method, can analyze variables in samples without known grouping and identify the principal components that best distinguish differences among samples (Fig. 3B). By analyzing the sample score chart using PCA for the entire dataset (Fig. 3C), PC1 represents 27.07% of variable differences, PC2 represents 14.50%, together accounting for 41.57% of total variance. Clear separation trends between sample groups were observed, with good clustering of replicates within each group, indicating significant differences among samples and credible data processing results. In this study, the metabolite data of the samples were analyzed from multiple angles using univariate and multivariate statistical analysis to accurately mine differential metabolites. The PLS-DA and OPLS-DA models were established and verified through 200 permutations to obtain nine comparison groups. Group 3 compared different periods of strain HY17 mono-culture: Ch-3d_vs_Ch-6d, Ch-6d_vs_Ch-10d, and Ch-3d_vs_Ch-10d. Six groups compared co-cultured samples for different periods: Ch-3d_vs_CO-3d, HM-3d_vs_CO-3d, Ch-6d_vs_CO-6d, HM-6d_vs_CO-6d, Ch-10d_vs_CO-10d, HM-10d_vs_CO-10d). The differences between the metabolite groups in different samples were significant, indicating that the model had good predictive ability and reliability with statistically significant differential metabolites obtained.

2.4 Dynamic analysis of differential metabolites in *A. sophora* HY17 mono-cultures

In order to understand the changes in secondary metabolites of *A. sophora* HY17 during growth and development, we screened differential metabolites using VIP values ($VIP > 1$) obtained from a combined OPLS-DA model and FC values ($\text{foldchange} \geq 2$ and $\text{foldchange} \leq 0.5$) from univariate analysis as criteria. We also compared the metabolites of *A. sophora* HY17 cultured alone at three time points using pairwise ensemble. It was found that *A. sophora* HY17 had the highest number of differential metabolites (146 species) when cultured alone at 3 dpi and 10 dpi. Fifteen differential metabolites were common across all three sets, while there were 17, 29, and 16 unique differential metabolites in the Ch-3d_vs_Ch-6d, Ch-6d_vs_Ch-10d, and Ch-3d_vs_Ch-10d sets respectively (Fig. 4A). In *A. sophora* HY17

culture, 6-Hydroxynicotinic acid and 3',7-dihydroxy-4'-methoxyflavone were significantly up-regulated in the early to middle stage (Fig. 4B). Camaldulenic acid was significantly down-regulated. Metabolites such as Chalcone were significantly up-regulated from the middle to late stage (Fig. 4C). Luteolin-7-O-glucoside (Cynaroside) and 5-O-Galloyl-D-hamamelose were significantly down-regulated. From the perspective of the whole growth cycle (Fig. 4D), 6-Hydroxynicotinic acid showed the largest increase with a fold change of 15.95. Luteolin-7-O-glucoside (Cynaroside) was the main down-regulated metabolite, with a fold change of -15.62. In conclusion, during the growth and development of *A. sophora* HY17, key secondary metabolites include 6-Hydroxynicotinic acid, 3',7-dihydroxy-4'-methoxyflavone, Camaldulenic acid, Chalcone and Cynaroside; however, most differential metabolites were down-regulated.

2.5 Metabolic dynamics during the interaction between *A. sophora* HY17 and *B. cinerea* HM1.

In order to investigate the dynamic changes of metabolites during the co-culture process of *A. sophora* HY17 and *B. cinerea* HM1, we used *A. sophora* HY17 monoculture and *B. cinerea* HM1 alone as controls, with co-culture serving as the experimental group. Metabolic profiles were examined at 3 dpi, 6 dpi, and 10 dpi for screening differential metabolites.

Compared to *A. sophora* HY17 mono-culture, Ch-3d_vs_CO-3d exhibited significant up-regulation of 37 metabolites and significant down-regulation of 45 metabolites at 3 dpi (Fig. 5A). Among them, flavonoids such as 3',7-dihydroxy-4'-methoxyflavone and Chalcone were significantly up-regulated, while Quercetin-5-O- β -D-glucoside and other substances were significantly down-regulated (Fig. 5B). At 6 dpi of culture, Ch-6d_vs_CO-6d showed significant up-regulation of 44 metabolites and significant down-regulation of 31 metabolites (Fig. 5C), with Chalcone and other substances being significantly up-regulated while 10-formyltetrahydrofuran was significantly down-regulated (Fig. 5D). At the end of the experiment on day ten, Ch-10d_vs_CO-10d showed twenty-seven significantly increased metabolites and eleven that had been decreased (Fig. 5E). Among them, the substance called 2,4,8-trihydroxy-1-tetralone was found to be notably increased, and those which had been decreased included 10-formyltetrahydrofuran and 7-C-Glucosylcoumarin (Fig. 5F).

The control group consisted of *B. cinerea* HM1 culture alone. At 3 dpi, HM-3d_vs_CO-3d (Fig. 6A) revealed significant up-regulation of 54 metabolites and down-regulation of 39 metabolites. Notably, Sophoridine, Lupanine, and 14 α -Hydroxymatrine were significantly up-regulated, while 6-Hydroxynicotinic acid was significantly down-regulated (Fig. 6B). At 6 dpi, HM-6d_vs_CO-6d (Fig. 6C) showed significant up-regulation of 57 metabolites and down-regulation of 34 metabolites. Among them, Lemnaine, Lupanine, Lehmennine, Sophocarpine, and Sophoridine were significantly up-regulated; whereas 10-Formyltetrahydrofuran was significantly down-regulated (Fig. 6D). At 10 dpi, HM-10d_vs_CO-10d (Fig. 6E) set revealed significant up-regulation of 65 metabolites and down-regulation of 13 metabolites. Notably, Sophoridine, Lehmennine, Lemnaine, Isomatrine, Sophocarpine, Matrine and other substances were significantly up-regulated; whereas 7-C-Glucosylcoumarin was significantly down-regulated (Fig. 6F).

Interestingly, a sustained and significant upregulation of alkaloids was observed when *B. cinerea* HM1 mono-culture was used as the control but no significant difference was found when *A. sophora* HY17 mono-culture was used as the control. This suggests that *A. sophora* HY17 has the capability to produce a variety of alkaloids along with potential antibacterial effects.

2.6 Relevant quantitative analysis of differential metabolites

To determine the relative quantitative changes of differential secondary metabolites in each category, metabolites with Log2FC > 10 from the co-culture screening will be selected for quantitative analysis. The screened substances include 14 alkaloids, 8 flavonoids, 5 terpenoids, 4 phenolic acids, 1 lignans and coumarin, and 2 other classes. A cluster heat map (Fig. 7) will be generated.

The analysis of alkaloid differential metabolites revealed that Lemnaine, Isomatine, Lupanine, Lehmannine, N-Oleoyethanolamine, Matrine, Sophocarpine, Sophoridine, 14 α -Hydroxymatine and Sophoranol exhibited high levels in the monoculture of *A. sophora* HY17, particularly on the 3rd day. These levels gradually decreased with prolonged culture time but showed an increasing trend during the late co-culture period. Conversely, the contents of 10-Formyltetrahydrofuran and 3-Hydroxyanthranilic acid were initially high in *A. sophora* HY17 but displayed a decreasing tendency in the late co-culture stage. Furthermore, while the content of 2-Oxo-3,4-dihydro-1H-quinoline and 3-carboxylic acid was low in *A. sophora* HY17 monoculture conditions; it increased over time during co-cultivation. Additionally observed was a higher content of 6-Hydroxynicotinic acid during *B. cinerea* HM1 monoculture stage which subsequently decreased during cocultivation. The metabolism of flavonoids exhibited significant differences, with the levels of Chalcone and 3',7-dihydroxy-4'-methoxyflavone showing a substantial increase during co-culture compared to monoculture of *A. sophora* HY17. Furthermore, at the 10th day of co-culture, there was a significant elevation in the content of Luteolin-7-O-glucoside (Cynaroside) when compared to *A. sophora* HY17 monoculture; whereas, at the 3rd day of co-culture, there was a notable decrease in Quercetin-5-O- β -D-glucoside content relative to *A. sophora* HY17 monoculture. Additionally, Myricetin-3-O-sulfonate, 6-C-methylQuercetin-3-O-Rutinoside, Vitexin-7-O-(6"-Feruloyl)glucoside and Daidzein-7-O-apiosyl(1 $\rightarrow$ 6) glucoside were found to have higher concentrations in *A. sophora* HY17 monoculture and experienced a significant increase during co-cultivation as compared to *B. cinerea* HM1 monoculture.

Terpenoids 2,3-Dihydroxyoleana-11,13(18)-dien-28-oic acid (Camaldulenic acid) and Olean-13(18)-en-3-one were found to have lower levels in the monoculture of *A. sophora* HY17 but higher levels in the monoculture of *B. cinerea* HM1. However, their contents significantly increased compared to that of *A. sophora* HY17 after co-culture. The contents of Epinootkatol and 2-methyl-2-vinyl-3-isopropenyl-5-isopropylidene cyclohexanol were observed to be higher in the monoculture of *A. sophora* HY17 and showed a significant increase during co-culture when compared with *B. cinerea* HM1 monocultivation. The levels of phenolic acids, including 3-[(1-Carboxyvinyl)oxy]benzoic acid, 5-O-Galloyl-D-hamamelose, 1-O-Galloyl-D-glucose and 3-O-Galloyl-D-glucose, were found to be increased in co-

culture compared to *A. sophora* HY17. Among them, cinnamoyl- β -D-glucose exhibited the highest levels on the 10th day of co-culture. Furthermore, the content of 7-C-Glucosylcoumarin was higher in *B. cinerea* HM1 monoculture but decreased during co-culture. The content of 2,4,8-trihydroxy-1-tetralone was significantly up-regulated in co-culture compared with *A. sophora* HY17 but still lower than that of *B. cinerea* HM1. Additionally, the content of Senkyunolide F decreased in co-culture compared with *A. sophora* HY17 monoculture; however, it showed an obvious trend towards upregulation when compared with *B. cinerea* HM1 monoculture.

2.7 Enrichment analysis of differential metabolites was performed using the KEGG database

In order to gain insights into the underlying pathways associated with differentiated metabolites, we performed enrichment analysis on the KEGG annotation results for metabolites exhibiting significant differences (Fig. 8). Metabolic pathways that were most significantly enriched included Ubiquinone and other terpenoid-quinone biosynthesis, Benzoate degradation, ABC transporters, Glutathione metabolism, Histidine metabolism, and Biosynthesis of cofactors in co-cultured samples at 3 dpi compared to control samples at 3 dpi. Additionally, D-Amino acid metabolism emerged as the most significantly enriched pathway in HM-3d_vs_CO-3d comparison. This comparison also revealed enrichment in Dioxin degradation, Lysine degradation, Aminobenzoate degradation, Biosynthesis of amino acids and various alkaloids, Metabolic pathways, Microbial metabolism in diverse environments and Biosynthesis of secondary metabolites. The most significantly enriched metabolic pathway in the co-culture at 6 dpi, Ch-6d_vs_CO-6d, was identified as Microbial metabolism in diverse environments. Additionally, this co-culture exhibited involvement in various biosynthetic processes such as benzoate degradation, cofactor biosynthesis, lysine degradation, ubiquinone and other terpenoid-quinone biosynthesis, aminobenzoate degradation, toluene degradation, and aromatic compound degradation. In contrast, the key pathway involved in HM-6d_vs_CO-6d was Biosynthesis of secondary metabolites. Furthermore, D-amino acid metabolism, lysine degradation pathways were also observed along with metabolic pathways related to benzoate degradation and amino acid biosynthesis including siderophore group nonribosomal peptides. The primary metabolic pathway involved in the metabolite set of Ch-10d_vs_CO-10d at 10 dpi in coculture is Phenylalanine metabolism. It also encompasses Glycine, serine and threonine metabolism, Microbial metabolism in diverse environments, Tyrosine metabolism, Biosynthesis of cofactors and Degradation of aromatic compounds. The most significantly enriched pathway in the HM-10d_vs_CO-10d set is Phenylalanine metabolism. Additionally included are Microbial metabolism in diverse environments, Bisphenol degradation, Benzoate degradation, and Tyrosine metabolism. Furthermore, Ubiquinone and other terpenoid-quinone biosynthesis as well as Degradation of aromatic compounds contribute to this enrichment along with Biosynthesis of terpenoid-Quinone biosynthesis secondary metabolites.

Overall, the biosynthetic pathways of secondary metabolites were consistently and significantly enriched in co-cultures, regardless of whether they were cultured with strain HY17 alone or with *Botryospora*

cinesulfum alone as controls. There was a significant enrichment in phenylalanine metabolism at 6 and 10 dpi.

2.8 Verification of antibacterial activity of differential metabolites

The inhibitory effect of the screened differential metabolites on *B. cinerea* HM1 growth was verified by conducting an antibacterial test using PDA medium containing 10 standard substances. Among these compounds, sophoridine, sophorocarpine, matrine, oleoethanolamine, 3-hydroxy-2-aminobranilic acid, chalcone, luteolin, daidzein, vitexin and 1-tetrahydronaphthone exhibited varying degrees of inhibition against *B. cinerea* HM1. The respective virulence regression equations were used to calculate the EC50 and EC90 values for different fungistatic active metabolites (Table 1, Fig. 10). Chalcone demonstrated the lowest inhibitory concentration among these compounds with EC50 and EC90 values of 60.03 and 118.16 µg/ml respectively.

Table 1
Inhibition rate of endophytic fungus HY17 against eight strains of pathogenic fungi

pathogenic fungi	Inhibition rate/%	
	simultaneous inoculation	delivery of HY17 for 3 days prior
Botrytis cinerea HM1	74.60 ± 2.75a	92.86 ± 2.38a
Rhizoctonia solani pn5-2	62.81 ± 1.34a	99.05 ± 0a
Fusarium avenacea YM1	54.18 ± 2.41b	96.30 ± 1.50a
Fusarium oxysporum pm29-3	49.49 ± 2.85bc	86.87 ± 2.86c
Colletotrichum siamense NX2-7	68.08 ± 2.09a	92.44 ± 1.26b
Fusarium tricuspidata pm36-8	49.52 ± 3.39bc	84.84 ± 0c
Clonostachys rosea pm34-5	53.22 ± 2.49bc	54.20 ± 2.64d
Pythium aphanidermatum pn8-3	46.42 ± 1.56c	86.12 ± 1.35c
Note: The data in the table are mean ± SD. Different lowercase letters in the same column indicate significant differences at the <i>P</i> < 0.05 level by Duncan's new complex range test.		

Table 2
Regression equations for the toxicity of ten active metabolites inhibiting *B.cinerea*

Compounds	Virulence regression equation	R2	EC50(μg/ml)	EC90(μg/ml)	95% Confidence Intervals
Sophoridine	$Y = 0.4902 * X - 44.04$	0.9801	191.84	273.44	0.4483–0.5321
Oleoylethanolamine	$Y = 0.3554 * X - 21.80$	0.9868	202.02	314.58	0.3336–0.3772
Sophocarpine	$Y = 0.3599 * X - 20.76$	0.9902	196.61	307.75	0.3409–0.3789
Matrine	$Y = 0.3695 * X - 16.12$	0.9791	178.94	287.21	0.3409–0.3981
3-Hydroxyanthranilic acid	$Y = 0.4791 * X - 43.43$	0.9685	195.01	278.51	0.4273–0.5308
Cynaroside	$Y = 0.4419 * X - 37.04$	0.9835	196.97	287.48	0.4015–0.4823
Daidzein	$Y = 0.3366 * X - 21.12$	0.9692	211.29	330.12	0.3048–0.3684
Chalcone	$Y = 0.6828 * X + 9.011$	0.9854	60.03	118.61	0.6387–0.7269
Vitexin	$Y = 0.3415 * X - 22.00$	0.9675	210.83	327.96	0.2974–0.3856
1-Tetralone	$Y = 0.2473 * X + 34.63$	0.9894	62.15	223.91	0.2244–0.2702

The results of high performance liquid chromatography (Fig. 10) showed that in the detection of alkaloids, matrine, sophoridine + sophoridine standard substances appeared at 9.338 min and 12.431 min, respectively (Fig. 10A), and the hypha extract of *A. sophora* HY17 appeared at 9.466 min and 12.506 min, respectively, which was similar to the chromatographic retention time of alkaloid standard substances and had good separation from other impurity peaks around it (Fig. 10B). In the detection of flavonoids, the peaks of vitexin, luteolin, daidzein and chalcone were 8.973, 9.622, 13.836 and 33.532 min, respectively (Fig. 10C), and the retention time of *A. sophora* HY17 mycelium extract with flavonoid standard was 8.960, 9.640, 13.880 and 34.700 min, respectively. In addition, the results of mass spectrometry also showed that the above substances were produced and accumulated (Supplement Fig. 1; Supplement Table 1); Therefore, it is determined that *A. sophora* HY17 can produce matrine, sophocarpine, sophoridine, vitexin, luteolin, daidzein and chalcone with antibacterial activity.

Discussion

Endophytic fungi in medicinal plants exhibit diverse and valuable biological activities, serving as a significant source of bioactive natural products with potential applications in medicine, food, and agriculture (Tyagi et al. 2021). A novel endophytic fungus, *Achaetomium sophora* HY17, was isolated from the medicinal plant *Sophora alopecuroides*. Through morphological and molecular biology analysis, it was identified as a new species. This is consistent with the results of previous studies (Tang et al. 2025). Studies have revealed that *Achaetomium* sp., a relatively uncommon genus of endophytic fungi, is found in most medicinal plants (Tyagi et al. 2021). However, there are limited reports on its metabolites and bacteriostatic substances. In this study, *A. sophora* HY17 exhibited broad-spectrum antifungal activity against eight pathogenic fungi, including *B. cinerea* HM1. Compared to simultaneous inoculation treatment, pre-inoculation of *A. sophora* HY17 three days before pathogenic fungi inoculation significantly enhanced its inhibitory effect on the pathogens. *B. cinerea* HM1, *R.solani* pn5-2, *F.avenacea* YM1 and *C.siamense* NX2-7 were all inhibited by more than 90%. Plant endophytic fungi mainly inhibit the infection and proliferation of pathogenic fungi through competition, antibiosis, and hyperparasitism (Vega 2018). Pre-inoculated endophytic fungi can occupy ecological sites, compete for nutrients, and produce antibiotics that inhibit the growth of pathogenic fungi effectively and showed a good biocontrol effect similar to Lu et al.'s research results (Oduro et al. 2023). As an endophytic fungus with environmental stability and strong colonization ability, *A. sophora* HY17 rapidly occupies nutrients and space while producing antibacterial substances that play a role in biocontrol.

In this study, the biocontrol fungus *A. sophora* HY17 was used to induce secondary metabolite accumulation in co-culture with *B. cinerea* HM1 as an external stimulus. Using UPLC-MS/MS metabolomics technology, we systematically explored the metabolic profile of *A. sophora* HY17 and identified alkaloids, flavonoids, phenolic acids, terpenes, lignans, and coumarin as the main metabolic substances similar to its host *Sophora alopecuroides* effective medicinal components (Wang et al. 2020). After model screening, several differential metabolites were obtained with alkaloids (14 types) and flavonoids (8 types) showing the most abundant species and significant relative quantitative changes. Among them, alkaloids such as sophoridine, sophocarpine, matrine, oleoylethanolamine, 3-hydroxyanthranilic acid were found to be the most abundant metabolites. Flavonoids like chalcone, cynaroside, daidzein, vitexin and other 1-tetralone played a crucial role in the interaction between *A. sophora* HY17 and *B. cinerea* HM1.

Alkaloids are secondary metabolites produced by living organisms during evolution to adapt to the natural environment and act as natural phytoalexins. Sun et al. (2022) discovered that matrine, oxymatrine, sophocarpine, and oxysophocarpine exhibited antibacterial effects against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, beta-hemolytic *Streptococcus*, and *Candida albicans*. In this study, alkaloid metabolites such as sophoridine, sophocarpine, matrine, oleoylethanolamine and 3-hydroxyanthranilic acid demonstrated potent antibacterial activity. Comparative analysis revealed a significant enrichment of alkaloid biosynthetic pathways during the interaction between *A. sophora* HY17 and *B. cinerea* HM1 with significantly up-regulated alkaloids in co-culture compared to *B. cinerea* HM1 alone. Among them were quinolone alkaloids including matrine, sophoridine, and sophocarpine. The effective alkaloid composition was consistent with that reported in

the host plant *Sophora sophora*, and both displayed broad-spectrum antibacterial activity (Pang et al. 2021). In the 1960s, researchers from the former Soviet Union isolated and extracted 18 flavonoids from licorice (Hulle et al.

1971). These flavonoids exhibit antibacterial activity against certain Gram-positive bacteria like *Staphylococcus aureus* and *Bacillus subtilis*, as well as Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* (Yong et al. 2006). During the interaction between *A. sophora* HY17 and *B. cinerea* HM1, eight differential metabolites of flavonoids were detected. Among them, Chalcone, 3',7-dihydroxy-4'-methoxyflavone, Quercetin-5-O- β -D-glucoside, Luteolin-7-O-glucoside showed a significant increase in content during co-culture compared to *A. sophora* HY17 mono-culture. The most significant change was observed in chalcone which exhibited a notable up-regulation trend at 3 dpi and an extremely high up-regulation at 6 dpi.

Additionally, *A. sophora* HY17 and *B. cinerea* HM1 exhibited the presence of terpenoids, phenolic acids, and coumarin compounds in their interaction. Previous studies have reported the isolation of 516 terpenoids from various plant endophytic fungi, which primarily have cytotoxic, antibacterial, and anti-inflammatory activities (Amirzakariya BZ, Shakeri A. 2022). Phenolic acids are significant natural secondary metabolites with considerable research value and application potential (Rashmi, H. B., Negi, P. S. 2020). Studies have revealed that phenolic acids extracted from plum fruits display inhibitory activities against six pathogenic fungi: *Xanthomonas vesicatoria*, *Botryosphaeria dothidea*, *Colletotrichum sisamense*, *Phytophthora capsici*, *Fusarium solani*, and *Fusarium graminearum* (Sehrawat et al. 2022). Coumarin compounds are widely present in medicinal plants and possess various biological activities, including information transmission, insecticidal, anti-cancer, and antibacterial properties (Ling et al. 2018). In addition to these compounds, this study also detected two other differential metabolites: 2,4,8-trihydroxy-1-tetralone and ligusticolactone F. Ligusticolide is a type of phthalide that can be extracted from medicinal plants like *Angelica sinensis* and *Ligusticum chuanxiong*. Pharmacological studies have demonstrated the wide range of pharmacological effects exhibited by Ligusticolide compounds such as antioxidative effect, anti-inflammatory activity, and anticoagulation (Li et al. 2023). Furthermore, this study found that 1-tetralone exhibits a potent inhibitory effect on *B. cinerea* HM1.

The phenylalanine metabolic pathway is crucial for the synthesis of various bioactive metabolites. In pathway, the key substrate is phenylalanine, catalyzed by key enzymes including phenylalanine ammonia-lyase (*PAL*), cinnamic acid 4-hydroxylase (*C4H*), and 4-coumaric acid coenzyme A ligase (*4CL*). The reaction produces intermediates such as coumaric acid, sinapic acid, trans-cinnamic acid, and ferulic acid. These intermediates are then transformed into chlorogenic acid, coumarin, trans-coumaryl-coa, and other compounds. Through multiple reaction pathways (Tao et al. 2017), these metabolites further generate secondary metabolites with pharmacological activity like flavonoids, alkaloids, lignin, and benzoate glycosides that play a vital role in fungal growth, development, and adaptation to the environment. Lu et al. (2019) sequenced the genome of the endophytic vine fungus *Alternaria* MG1 and identified that the phenylalanine metabolic pathway plays a crucial role in secondary metabolite synthesis. Twenty-one proteins encoded by multiple genes, catalyzing/supporting the biosynthesis of

flavonoids, alkaloids, and lignin in this pathway. KEGG enrichment analysis of differential metabolites in this study revealed significant enrichment of coumarin, flavonoids, and alkaloids in the phenylalanine metabolic pathway during *A. sophora* HY17 co-culture with *B. cinerea* HM1 at middle and late stages. Similarly, Chen et al. (2024) found that *Streptomyces virginiae* XDS1-5 had differential metabolites with antibacterial activity enriched in the phenylalanine metabolism pathway, such as indoleines. These findings suggest that the phenylalanine metabolic pathway may be important for synthesizing secondary metabolites like flavonoids and alkaloids during the interaction between *A. sophora* HY17 and *B. cinerea* HM1.

There are some limitations of the current study. The current study initially identified the major antimicrobial species of *A. sophora* HY17, however, its metabolic characteristics and antibacterial mechanisms remain largely unknown. In the next step, we will focus on the phenylalanine metabolic pathway and utilize whole genome and comparative transcriptome technology to identify key genes involved in alkaloid and flavonoid synthesis, thus elucidating the complete biosynthetic pathway of these compounds in *A. sophora* HY17. Overall, the current study provides a theoretical foundation for enhancing the production of antibacterial substances in *A. sophora* HY17 through genetic modification.

Conclusion

A. sophora HY17 exhibits broad-spectrum antibacterial activity and possesses significant biocontrol potential as a resource fungus. Co-culture and UPLC-MS/MS metabolomics analysis have preliminarily identified alkaloids and flavonoids as the main active substances produced during the interaction between *A. sophora* HY17 and *B. cinerea* HM1. Ten differential metabolites, including sophoridine, sophocarpine, matrine, oleoylethanolamine, 3-hydroxyanthranilic acid, chalcone, cynaroside, daidzein, vitexin and 1-tetralone were detected. These metabolites are primarily involved in Phenylalanine metabolism pathways. In conclusion, *A. sophora* HY17 is a natural valuable resource of novel antibacterial compounds with application prospects.

Declarations

Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Funding information

This work was funded by the Key Research and Development Project in Ningxia Province: Development and Application of Biological Control Agents for Grape Root Diseases in the Eastern Foothills of Helan Mountain (2023BCF01026).

Acknowledgments

First of all, I would like to express my deepest gratitude to my mentor, Gu. She is an admirable, responsible, and resourceful scholar who has provided me with invaluable guidance at every stage of the writing of this thesis. I would also like to thank all my laboratory friends who helped me to design and write this paper, for their encouragement and support.

References

1. Ahmad R, Lim CK, Marzuki NF, Goh YK, Baharum SN (2020) Metabolic Profile of *Scytalidium parasiticum*-*Ganoderma boninense* Co-Cultures Revealed the Alkaloids, Flavonoids and Fatty Acids that Contribute to Anti-*Ganoderma* Activity. *Molecules* 25(24). <https://doi.org/10.3390/molecules25245965>
2. Amirzakariya BZ, Shakeri A (2022) Bioactive terpenoids derived from plant endophytic fungi: An updated review (2011–2020). *Phytochemistry* 197(1). <https://doi.org/10.1016/j.phytochem.2022.113130>
3. An C, Ma S, Shi X, Xue W, Liu C, Ding H (2020) Diversity and Antimicrobial Activity of Endophytic Fungi Isolated from *Chloranthus japonicus* Sieb in Qinling Mountains, China. *Int J Mol Sci* 21(17):5958. <https://doi.org/10.3390/ijms21175958>
4. Chagas FO, Dias LG, Pupo MT (2013) A mixed culture of endophytic fungi increases production of antifungal polyketides. *J Chem Ecol* 39(10):1335–1342. <https://doi.org/10.1007/s10886-013-0351-7>
5. Chen HY, Liu TK, Shi Q, Yang XL (2019) Sesquiterpenoids and diterpenes with antimicrobial activity from *Leptosphaeria* sp. XL026, an endophytic fungus in *Panax notoginseng*. *Fitoterapia* 137:104243. <https://doi.org/10.1016/j.fitote.2019.104243>
6. Chen M, Jia F, Chen S, Zheng Y, Hu Y, Liu W, Liu C, Sun X, Lu J, Chen G (2024) *Streptomyces virginiae* XDS1-5, an antagonistic actinomycete, as a biocontrol to peach brown rot caused by *Monilinia fructicola*. *J Sci Food Agric* 104(12). <https://doi.org/10.1002/jsfa.13573>
7. Cui YM, Yu LJ, Ao MZ, Yang Y, Hu J (2006) Studies on flavonoids extraction technology from *Glycyrrhiza inflata* and their bacteriostatic activities. *J Chin Med Mater* 29(8):838–841. <https://pubmed.ncbi.nlm.nih.gov/17076245/>
8. Dinglasan JLN, Otani H, Doering DT, Udway D, Mouncey NJ (2025) Microbial secondary metabolites: advancements to accelerate discovery towards application. *Nat Rev Microbiol*. <https://doi.org/10.1038/s41579-024-01141-y>
9. Glass NL, Donaldson GC (1995) Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Appl Environ Microbiol* 61(4):1323–1330. <https://doi.org/10.1128/aem.61.4.1323-1330.1995>
10. Gokhale M, Gupta D, Gupta U, Faraz R, Sandhu SS (2017) Patents on Endophytic Fungi. *Recent Patents Biotechnol* 11(2):120–140. <https://doi.org/10.2174/1872208311666170215151834>

11. Helge BBD, Barbara BD, Regina HD (2002) Big effects from small changes: possible ways to explore nature's chemical diversity. *ChemBioChem* 3(7). [https://doi.org/10.1002/1439-7633\(20020703\)3:7<619::AID-CBIC619>3.0.CO;2-9](https://doi.org/10.1002/1439-7633(20020703)3:7<619::AID-CBIC619>3.0.CO;2-9)
12. Isabel V, Riccardo B, Rosa H, Enrique MGV, Sabrina S (2022) Role and genetic basis of specialised secondary metabolites in *Trichoderma* ecophysiology. *Fungal Biology Reviews* 39(0):83–99. <https://doi.org/10.1016/j.fbr.2021.12.004>
13. Ju MX, Zhang QC, Wang RT et al (2022) Correlation in endophytic fungi community diversity and bioactive compounds of *Sophora alopecuroides*. *Front Microbiol* 13. <https://doi.org/10.3389/fmicb.2022.955647>
14. LI Q, ZHANG YY, YANG ZY et al (2024) Establishment of an HPLC Method for Simultaneous Determination of Ten Flavonoids in Germinated Black Wheat. *ournal Food Sci Biotechnol* 43(10):109–119. <https://doi.org/10.12441/spyswjs.20240414001>
15. Li Q, Wan JB, Zhao L (2023) Research progress on the pharmacological activities of senkyunolides. *Acupunct Herb Med* 3(3):180–188. <https://doi.org/10.1097/hm9.0000000000000075>
16. Li X, Zhang., Zhao Y, Feng J, Chen Y, Li K, Zhang M, Qi D, Zhou D, Wei Y, Wang W, Xie J (2024) Biocontrol potential of volatile organic compounds produced by *Streptomyces corchorusii* CG-G2 to strawberry anthracnose caused by *Colletotrichum gloeosporioides*. *Food Chem* 437(Pt 2):137938. <https://doi.org/10.1016/j.foodchem.2023.137938>
17. Ling HP, Chen XQ, Xie SN, Chen YJ, She ZG, Lin YC, Tao YW (2018) Dihydroisocoumarin Compounds from Endophytic Fungi of *Rhizophora apiculata* and Its Antibacterial Activity. *Chin J Appl Chem* 35(6):708–713. <https://doi.org/10.11944/j.issn.1000-0518.2018.06.170281>
18. Liu ZP, Kang B, Duan XR, Hu Y, Li W, Wang C, Li DS, Xu N (2022) Metabolomic profiles of the liquid state fermentation in co-culture of *A. oryzae* and *Z. rouxii*. *Food Microbiol* 103(0):103966–103966. <https://doi.org/10.1016/j.fm.2021.103966>
19. Lu Y, Ye C, Che J, Xu XG, Shao DY, Jiang CM, Liu YL, Shi JL (2019) Genomic sequencing, genome-scale metabolic network reconstruction, and in silico flux analysis of the grape endophytic fungus *Alternaria* sp. MG1. *Microb Cell Fact* 18(1):0–0. <https://doi.org/10.1186/s12934-019-1063-7>
20. Mehrabi M, Asgari B, Zare R (2020) Description of *Allocanariomyces* and *Parachaetomium*, two new genera, and *Achaetomium aegilopis* sp. nov. in the Chaetomiaceae. *Mycological Progress* 19(12):1415–1427. <https://doi.org/10.1007/s11557-020-01636-x>
21. Oduro MD, Lowor ST, Bukari Y, Donkor JK, Minnah B, Nuhu AH, Dontoh D, Amadu AA, Ocloo A (2023) Cocoa-associated filamentous fungi for the biocontrol of aflatoxigenic *Aspergillus flavus*. *J Basic Microbiol* 63(11):1279–1292. <https://doi.org/10.1002/jobm.202300163>
22. Pang Z, Chen J, Wang T, Gao C, Li Z, Guo L, Xu J, Cheng Y (2021) Linking plant secondary metabolites and plant microbiomes: a review. *Front Plant Sci* 12:621276. <https://doi.org/10.3389/fpls.2021.621276>
23. Partida-Martinez LP, Hertweck C (2005) Pathogenic fungus harbours endosymbiotic bacteria for toxin production. *Nature* 437(7060):884–888. <https://doi.org/10.1038/nature03997>

24. Peng XY, Wu JT, Shao CL, Li ZY, Chen M, Wang CY (2021) Co-culture: stimulate the metabolic potential and explore the molecular diversity of natural products from microorganisms. *Mar Life Sci Technol* 3(3):363–374. <https://doi.org/10.1007/s42995-020-00077-5>
25. Rashmi HB, Negi PS (2020) Phenolic acids from vegetables: A review on processing stability and health benefits. *Food research international*. (Ottawa Ont) 136:109298. <https://doi.org/10.1016/j.foodres.2020.109298>
26. Rehner SA, Samuels GJ (1995) Molecular systematics of the Hypocreales: a teleomorph gene phylogeny and the status of their anamorphs. *Can J Bot* 73(1):816–823. <https://doi.org/10.1139/b95-327>
27. Romano S, Jackson SA, Patry S, Dobson ADW (2018) Extending the One Strain Many Compounds (OSMAC) Principle to Marine Microorganisms. *Mar Drugs* 16(7):244–244. <https://doi.org/10.3390/md16070244>
28. Sehrawat R, Rathee P, Akkol EK, Khatkar S, Lather A, Redhu N, Khatkar A (2022) Phenolic Acids - Versatile Natural Moiety with Numerous Biological Applications. *Curr Top Med Chem* 22(18):1472–1484. <https://doi.org/10.2174/1568026622666220623114450>
29. Sun XY, Jia LY, Rong Z, Zhou X, Cao LQ, Li AH, Guo M et al (2022) Research Advances on Matrine. *Frontiers Chem* 10:867318. <https://doi.org/10.3389/fchem.2022.867318>
30. Zhiyun TANG, LIU WANGWK GL, et al (2025) Optimization of culture conditions for the sophoridine producing new fungal species *Achaetomium* sophor strain HY17 isolated from seeds of *Sophora alopecaroides*. *Acta Prataculturae Smca* 34(10):151–163. <https://doi.org/10.11686/cyxb2024444>
31. Tao X, Fang Y, Huang MJ, Xiao Y, Liu Y, Ma XR, Zhao H (2017) High flavonoid accompanied with high starch accumulation triggered by nutrient starvation in bioenergy crop duckweed (*Landoltia punctata*). *BMC Genomics* 18(1). <https://doi.org/10.1186/s12864-017-3559-z>
32. Triastuti A, Haddad M, Barakat F, Mejia K, Rabouille G, Fabre N, Amasifuen C, Jargeat P, Vansteelandt M (2021) Dynamics of Chemical Diversity during Co-Cultures: An Integrative Time-Scale Metabolomics Study of Fungal Endophytes *Cophinforma mamane* and *Fusarium solani*. *Chem Biodivers* 18(2):e2000672. <https://doi.org/10.1002/cbdv.202000672>
33. Tu T, Meng K, Bai YG, Shi PJ, Luo HY, Wang YR, Yang PL, Zhang YH, Zhang W, Yao B (2013) High-yield production of a low-temperature-active polygalacturonase for papaya juice clarification. *Food Chem* 141(3):2974–2981. <https://doi.org/10.1016/j.foodchem.2013.05.132>
34. Tyagi G, Kapoor N, Chandra G, Gambhir L (2021) Cure lies in nature: medicinal plants and endophytic fungi in curbing cancer. *3 Biotech* 11(6):263. <https://doi.org/10.1007/s13205-021-02803-x>
35. van Hulle C, Braeckman P, Vandewalle M (1971) Isolation of two new flavonoids from the root of *Glycyrrhiza glabra* var. *typica*. *Planta Med* 20(3):278–282. <https://doi.org/10.1055/s-0028-1099705>
36. Vega FE (2018) The use of fungal entomopathogens as endophytes in biological control: a review. *Mycologia* 110(1):4–30. <https://doi.org/10.1080/00275514.2017.1418578>
37. Wang RT, Zhang QC, Ju MX, Yan SY, Zhang QQ, Gu PW (2022) The Endophytic Fungi Diversity, Community Structure, and Ecological Function Prediction of *Sophora alopecuroides* in Ningxia.

- China Microorganisms 10(11):2099–2099. <https://doi.org/10.3390/microorganisms10112099>
38. Wang R, Deng X, Gao Q, Wu X, Han L, Gao X, Zhao S, Chen W, Zhou R, Li Z, Bai C (2020) *Sophora alopecuroides* L.: An ethnopharmacological, phytochemical, and pharmacological review. Journal of ethnopharmacology 248, 112172. <https://doi.org/10.1016/j.jep.2019.112172>
39. Wang XW, Houbraken J, Groenewald JZ, Meijer M, Andersen B, Nielsen KF, Crous PW, Samson RA (2016) Diversity and taxonomy of Chaetomium and chaetomium-like fungi from indoor environments. Stud Mycol 84(1):145–224. <https://doi.org/10.1016/j.simyco.2016.11.005>
40. Wang XW, Yang FY, Meijer M, Kraak B, Sun BD, Jiang YL, Wu YM, Bai FY, Seifert KA, Crous PW, Samson RA, Houbraken J (2019) Redefining Humicola sensu stricto and related genera in the Chaetomiaceae. Stud Mycol 93:65–153. <https://doi.org/10.1016/j.simyco.2018.07.001>
41. White TC, Bruns TD, Lee S, Taylor FJR, White TJ, Lee SH, Taylor DL, Taylor JS (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR protocols: a guide to methods and applications. null 0 (0), 0–0. <https://doi.org/null>
42. Whitt JA, Shipley SM, Newman DJ, Zuck KM (2014) Tetramic Acid Analogues Produced by Coculture of Saccharopolyspora erythraea with Fusarium pallidoroseum. J Nat Prod 77(1):173–177. <https://doi.org/10.1021/np400761>
43. Zhao L, Meng K, Bai YG, Shi PJ, Huang HQ, Luo HY, Wang YR, Yang PL, Song W, Yao B (2013) Two Family 11 Xylanases from *Achaetomium* sp. Xz-8 with High Catalytic Efficiency and Application Potentials in the Brewing Industry. J Agric Food Chem 61(28):6880–6889. <https://doi.org/10.1021/jf4001296>
44. Zhu XW, Zhou DL, Liang FY, Wu ZH, She ZG, Li CY (2017) Penochalasin K, a new unusual chaetoglobosin from the mangrove endophytic fungus Penicillium chrysogenum V11 and its effective semi-synthesis. Fitoterapia 123(0):23–28. <https://doi.org/10.1016/j.fitote.2017.09.016>

Figures

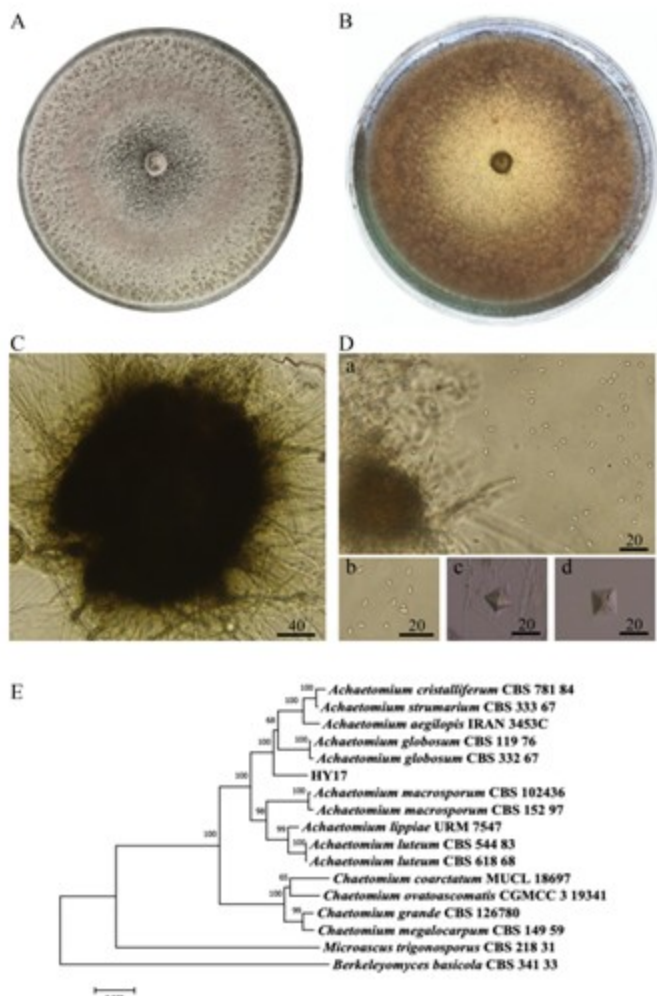


Figure 1

Morphological characteristics and molecular identification of the endophytic fungus HY17 isolated from *Sophora alopecuroides* based on *ITS-LSU-TUB* polygene tandem.

Note: A and B: Colony of fungus HY17; C: The microscopic morphology of ascus of fungus HY17; D: Spores of fungus HY17 (ab. ascospore, cd. Chlamydospore; Scale unit: μm); E: Fungus HY17 polygenic phylogenetic tree (*ITS-LSU-TUB*).

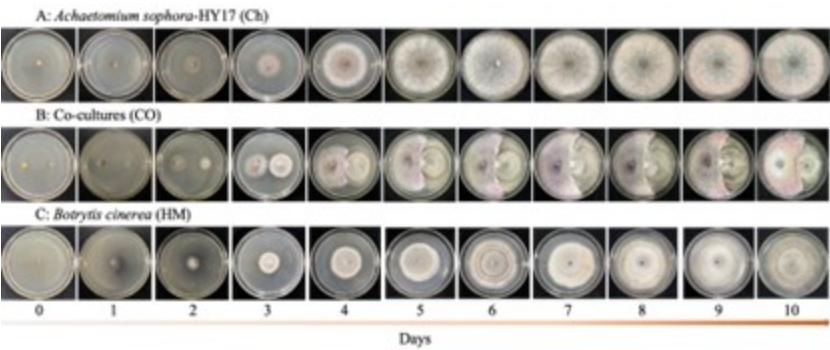


Figure 2

Morphological alterations of *A. sophora* HY17 co-cultured with *B. cinerea* HM1 for 0-10 days

Note: A: Monoculture of *A. scaphora* HY17 (Ch); B: Co-cultures of *A. sophora* HY17 and *B. cinerea* HM1 (CO); C: Monoculture of *B. cinerea* HM1 (HM).

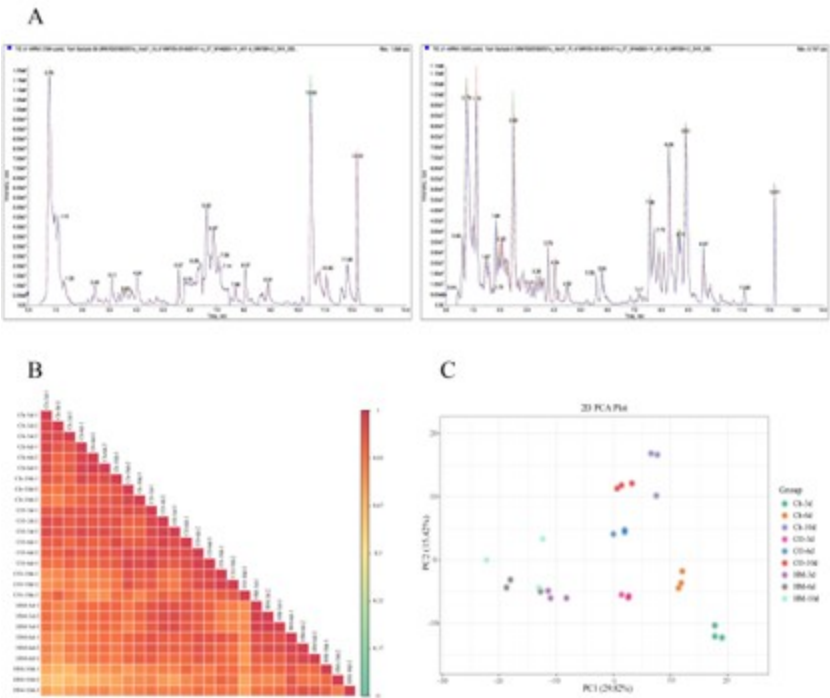


Figure 3

Detection and analysis of metabolomic-like essence-spectrum in population samples

Note: A: The essence spectrum of the QC sample was analyzed to detect the overlap map of total ion chromatogram (TIC); B: Repetitive evaluation of correlation in test samples; C: Principal Component Analysis (PCA) score plot for the entire sample set.

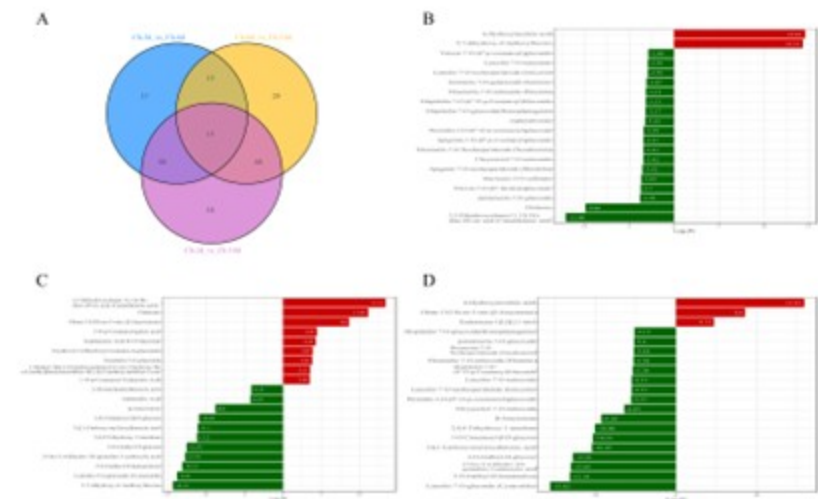


Figure 4

Dynamic alterations in metabolite profiles of *A. sophora* HY17 under isolated culture conditions

Note: A: Venn diagram illustrating the distinct metabolites of *A. sophora* HY17 cultured in isolation at various time points; B-D: Comparisons of metabolite profiles between Ch-3d and Ch-6d (B), Ch-6d and Ch-10d (C), and Ch-3d and Ch-10d (D) were conducted.

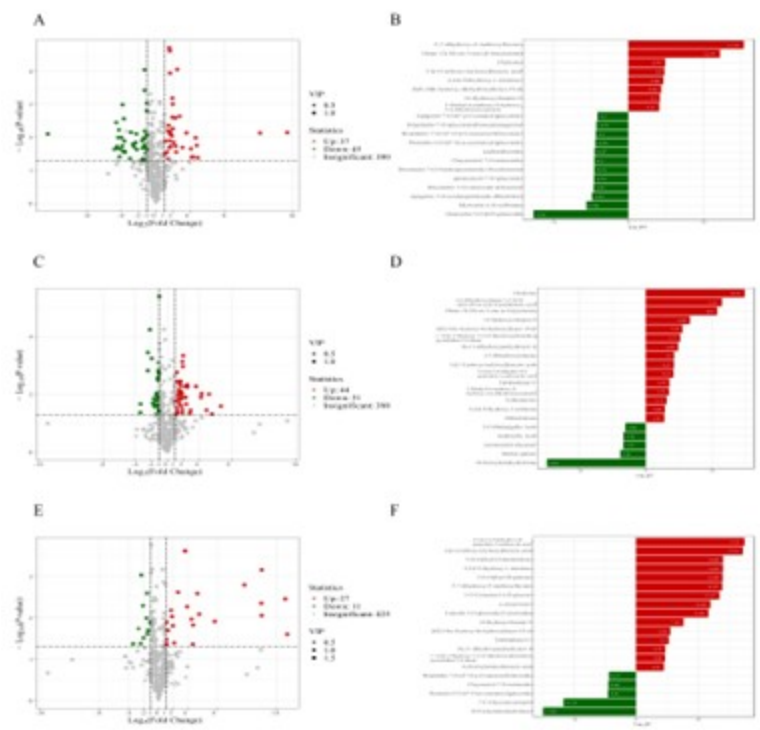


Figure 5

Comparison of differential metabolites between *A. sophora* HY17 monoculture and co-culture

Note: A, C and E: Volcanic heatmaps depicting differential metabolites of Ch-3d_vs_CO-3d (A), Ch-6d_vs_CO-6d (C), Ch-10d_vs_CO-10d (E); B, D and F: Bar charts depicting the top 20 differential metabolites of Ch-3d_vs_CO-3d (B), Ch-6d_vs_CO-6d (D), and Ch-10d_vs_CO-10d (F).

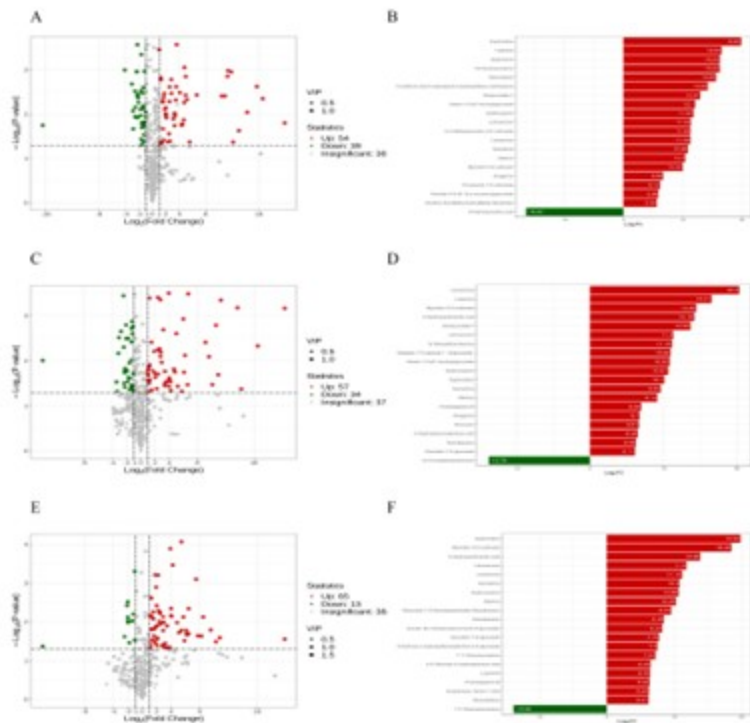


Figure 6

Comparison of differential metabolites between *B. cinerea* HM1 monoculture and co-culture

Note: A, C and E: Volcanic heatmaps depicting differential metabolites of HM-3d_vs_CO-3d (A), HM-6d_vs_CO-6d (C), HM-10d_vs_CO-10d (E); B, D and F: Bar charts depicting the top 20 differential metabolites of HM-3d_vs_CO-3d (B), HM-6d_vs_CO-6d (D), and HM-10d_vs_CO-10d (F).

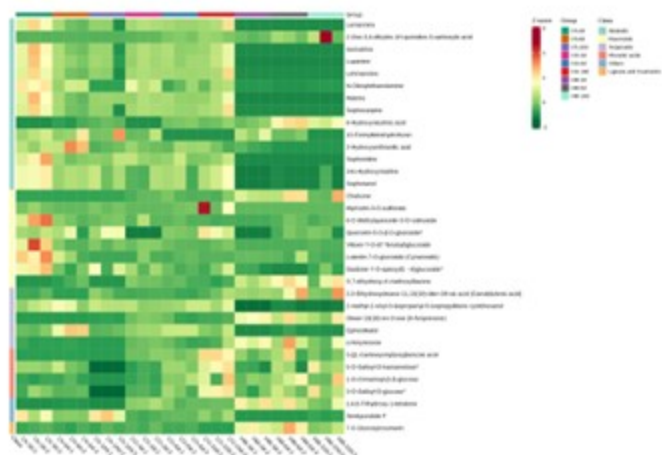


Figure 7

Heat map illustrating the results of relative quantitative analysis for differential metabolites (Log2FC>10).

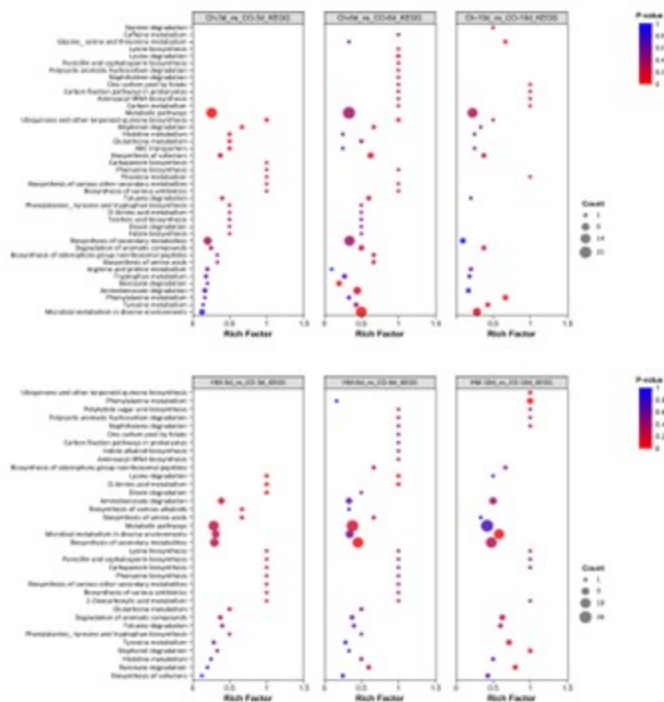


Figure 8

KEGG classification and enrichment analysis of differential metabolites

Note: The horizontal coordinate represents the Rich Factor corresponding to each pathway, while the vertical coordinate denotes the path name (sorted by P-value). The color of the dots reflects the magnitude of the P-value, with red indicating a higher level of significance in enrichment. Additionally, the size of the dots indicates the extent to which differential metabolites are enriched.

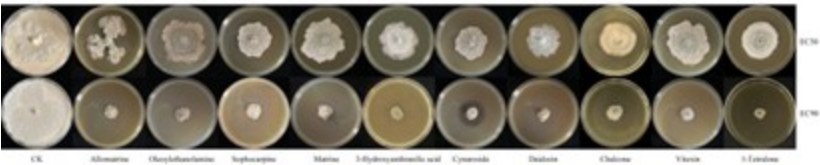


Figure 9

Inhibition of *B. cinerea* by active metabolites

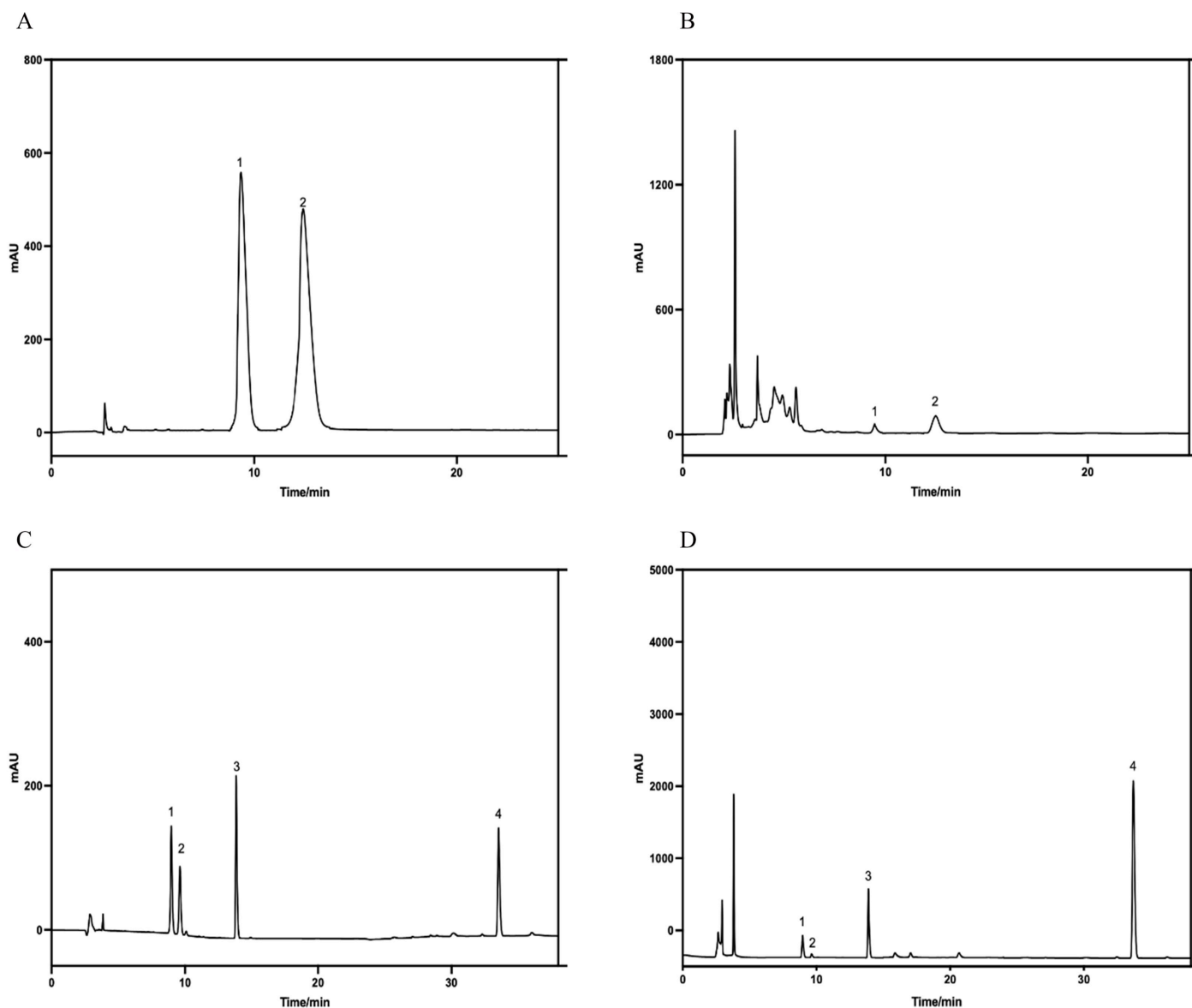


Figure 10

HPLC images of three alkaloid and four flavonoid standards and mycelium extracts of *A. sophora* HY17

Note: A: Three alkaloid standards (1: matrine; 2: sophocarpine and sophoridine); B: A. Detection of alkaloids in hyphal extracts of sophora HY17; C: four flavonoid standards (1: vitexin; 2: luteolin; 3: daidzein; 4: chalcone); D: A. Flavonoid detection of hyphal extracts from sophora HY17.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [ST1.docx](#)