

The exogenous salicylic acid-induced changes of metabolomic profile and insect resistance in *Monotropa uniflora*

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Abstract

Monotropa uniflora, a medicinal and edible herb, exhibits antitussive activity and tonifying efficacy on pulmonary deficiency. In the present study, we aimed to assess the impact of exogenous salicylic acid (SA) on the feeding behaviour of *Spodoptera exigua* and on the metabolomic profile of *M. uniflora*. The results showed that the application of exogenous SA on *M. uniflora* plants significantly reduced the feeding activities of *S. exigua* on *M. uniflora* (MuT), when comparing with those on the *M. uniflora* controls (MuCK) ($P < 0.05$). Based on metabolomic analysis using liquid chromatography-tandem mass spectrometry (LC-MS/MS), a total of 1,644 metabolites in *M. uniflora* were identified and annotated in the Human Metabolome Database (HMDB). Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) revealed significant differences between MuCK and MuT samples. Following SA induction, 321 compounds were upregulated, while 137 compounds were downregulated in *M. uniflora*. Secondary metabolic classification indicated that terpenes, phenolic acids and their derivatives, and flavonoids were the major categories. Among these compounds, the upregulation of caffeic acid, gibberellins A86, and gibberellins A7, as well as the downregulation of β -ionone, linalool, malvidin, and peonidin 3-rhamnoside 5-glucoside, were suggested to play roles in enhancing the resistance of *M. uniflora* to diseases and herbivores. The enrichment of metabolic pathways such as monoterpenoid biosynthesis, limonene and pinene degradation, and phenylalanine metabolism was determined to be critical for the improvement of *M. uniflora* resistance. Furthermore, the 321 upregulated metabolites were highly enriched in pathways including linoleic acid metabolism, while the 137 downregulated metabolites were significantly enriched in pathways such as nucleotide metabolism, cyanoamino acid metabolism, and arginine biosynthesis. These enrichments are also suggested to contribute to the enhanced resistance of *M. uniflora*.

1. Introduction

Monotropa uniflora L., which belongs to the genus *Monotropa* in the family Ericaceae (Pradhan, 2015). *M. uniflora* is a wild herbaceous angiosperm not typically cultivated in horticultural settings. It is an important traditional Chinese medicine with extremely high medicinal and economic value. *M. uniflora*, a plant traditionally used in both culinary and medicinal applications, is reported to exhibit expectorant and antitussive effects. The plant has a variety of pharmacological activities, which can be used to treat diseases, including cough and deficiency. *M. uniflora* thrives in dark and humid environments at altitudes ranging from 800 to 3800 meters. This unique species cannot photosynthesize due to the lack of chlorophyll in *M. uniflora*, and only relies on absorbing decomposed soil to obtain nutrients (Young et al., 2002; Shen et al., 2012), hence being known as ghost flower. *M. uniflora* is a national second-class protected plant in China. It exhibits a broad global distribution, primarily inhabiting regions in Japan, Russia, North America, India, and Korea. Within China, its presence is primarily noted in the provinces of Yunnan, Guizhou, and Guangxi and other provinces (Liu et al., 2020).

Like *M. uniflora*, *Vaccinium dunalianum* also belongs to the Ericaceae family. Some previous studies have shown that many chemicals in *V. dunalianum* have biological and pharmacological activities, and hold

great promise for drug development and biopesticide applications. (Raudone et al., 2019; Makinen et al., 2020; Vilkickyte and Raudone 2021). However, for *M. uniflora*, little is known about the diverse chemical components and pharmacological mechanism because it is difficult to obtain the plant of *M. uniflora* growing under the demanding conditions difficult to meet. The chemical components of *M. uniflora* have not been well-documented. Therefore, researching on the chemical composition of *M. uniflora* and understanding the control strategies of pests and diseases on *M. uniflora* are critically important, as it contributes to a deeper understanding of this species and provides novel approaches for its cultivation.

In recent years, metabolomics and other biotechnologies, as prominent research approaches, have been widely applied in studies on plant diseases and pests (Adeniji et al., 2020; Alawiye and Babalola, 2020; Mashabela et al., 2022). For example, metabolomic analysis revealed significant metabolic alterations in cucumber plants infected with *Cucumber mosaic virus* (CMV) and treated with entomopathogenic fungi. These changes primarily involved amino acids, organic acids, and phenylpropanoid metabolites, offering novel insights into cucumber defense mechanisms against CMV (Shaaan et al., 2022). *Tilletia controversa* infection significantly enhances the expression of prostaglandins (PGs) and 9-hydroxyoctadecadienoic acids (9-HODEs) in winter wheat while altering the concentrations of cucurbitacin and octadecatrienoic acid, thereby impairing plant defense responses. Concurrently, eight associated metabolic pathways in winter wheat are activated, including phenylalanine metabolism and arginine metabolism (Ren et al., 2021).

Salicylic acid (SA), a phenolic acid metabolite commonly found in plants, is synthesized by plants, and transported through the phloem. As a crucial plant hormone, it participates in signal transduction pathways and plays a significant role in enhancing plant resistance (Wildermuth et al., 2001; Vlot et al., 2009). The application of oligogalacturonides to *Arabidopsis thaliana* can activate the SA signaling pathway, reduce the feeding behavior of *Myzus persicae*, and thereby enhance resistance to this pest (Silva-Sanzana et al., 2022). Chlorogenic acid (CGA) treatment of peach fruit enhances the activity of defense-related enzymes and increases the expression of key genes in the SA signaling pathway. This indicates that CGA treatment is an effective method for controlling postharvest blue mold in peaches, and that the activation of the SA signaling pathway may play a significant role in the CGA-induced resistance (Jiao et al., 2018). In addition, many studies have shown that the application of exogenous SA significantly enhances both disease resistance and pest resistance in plants. Exogenous SA significantly enhanced resistance to *Glomerella cingulata* leaf spot (GLS) in apple, and increased the activities of antioxidant enzymes (CAT, SOD, POD, PAL, PPO) (Zhang et al., 2016). The application of exogenous SA was found to effectively inhibit the growth and reproduction of *Toxoptera aurantii* on tea plants. Treatment with low concentrations of SA resulted in a significant reduction in pest infestation levels. Concurrently, a significant enhancement in the activity of defense-related enzymes, such as polyphenol oxidase, within the tea plants was observed (Jiang et al., 2024). A significant reduction in both the incidence and diameter of powdery mildew lesions on citrus fruits was observed following exogenous SA application (Wang et al., 2020). The application of exogenous SA induced the accumulation of specific secondary metabolites in *Panax vietnamensis*, particularly coumarins and anthocyanins, resulting in augmented resistance to the pathogenic fungus *Neofusicoccum ribis* (Hou et al., 2024).

However, little is known about whether exogenous SA can significantly alter the metabolites to synthesize new plant metabolites and thereby enhance plant resistance against pests and diseases. Especially, the metabolomic changes in plants treated with exogenous SA are still unclear.

Up to date, the effects of exogenous SA induction on the metabolite profiles and resistance of *M. uniflora* against *Spodoptera exigua* have not been investigated. The present study aims to analyze the differences in metabolite profiles between exogenous SA-induced *M. uniflora* and untreated *M. uniflora*. Subsequently, these differential metabolites were utilized for metabolomics and metabolic pathway analysis to understand the defense response of exogenous SA-induced *M. uniflora* to pests and diseases, and to explore its resistance mechanisms.

2. Materials and methods

2.1 Cultivation and exogenous SA treatment of *M. uniflora*

The *M. uniflora* plants used in the experiment were purchased from a nursery garden located at 26°33'N, 100°10'E in Heqing County, Yunnan Province. The area has a plateau monsoon climate with dry winters and cool summers. The average altitude is 2196 m. The average regional yearly precipitation is 959.4 cm, the average annual temperature is 13.5°C, and the average annual relative humidity is 52%. All selected samples of *M. uniflora* have been growing for at least four months (Xiao et al., 2007).

To study exogenous SA-induced changes in resistance and metabolome of *M. uniflora*, only healthy, and vigorous plants were selected. The plants were controlled at room temperature during the day and around zero degrees at night, and normal watering and fertilization were applied before the treatment. Ten plants of *M. uniflora* were sprayed with 0.01 mol/l SA in 10% (V/V) ethanol-water (MuT) once daily for six days, while another ten plants of *M. uniflora* treated with 10% (V/V) ethanol-water were used as the control (MuCK). Each group was set up in 6 replicates. Then, the entire *M. uniflora* plants were removed and weighed. All *M. uniflora* samples were cleaned with sterile DI water and stored in liquid nitrogen at -80°C in centrifuge tubes. All of 12 samples were prepared and divided into 2 groups, each containing 6 parts. Two biological replicates were done for each treatment for metabolomic analysis and twelve *M. uniflora* samples were numbered as MuCK1, MuCK2, MuCK3, MuCK4, MuCK5, MuCK6, MuT1, MuT2, MuT3, MuT4, MuT5, and MuT6, corresponding to six healthy (control) and six SA-induced *M. uniflora* plants.

2.2 *Spodoptera exigua*

S. exigua larvae used in our study was purchased from the Keyun Biotechnology Co. in Jiyuan City, Henan Province (34°52'N, 112°38'E). The area has a plateau monsoon climate with dry winters and cool summers. The average altitude is 1711 m. The feeding temperature for *S. exigua* is 28°C, with a humidity of 65%. Disease-free and healthy *S. exigua* larvae with same body conditions were selected for the experiment.

2.3 Bioassays of plant height, stem diameter, and feeding amount

To investigate the effects of exogenous SA on the growth of *M. uniflora* and the feeding behavior of *S. exigua*, a series of experiments were conducted. Ten *M. uniflora* plants exhibiting vigorous growth were selected for the experiment. Following treatment with exogenous SA, stem diameter and plant height were measured daily for six days using vernier calipers. Five replicates were used for both the treatment and control groups. After the six-day treatment, plants were harvested, rinsed with deionized water, air-dried, and weighed to determine fresh weight. Plant material was then fragmented. Six petri dishes were prepared, each containing equal weight of plant material and ten healthy third-instar *S. exigua* larvae with approximately equal body weight. The ten larvae were allowed to feed on the MuT fragments in a petri dish and another ten larvae were allowed to feed on the MuCK fragments in another petri dish (controls). Their daily consumption was recorded for six days. Consumption was calculated as $m \text{ (mg)} = (m_2 - m_1) \text{ mg}$, where m represents the consumed weight of *M. uniflora*, m_1 represents the initial weight, and m_2 represents the final weight after feeding. All data were subjected to statistical analysis using SPSS to determine significance.

2.4 Extraction of Metabolites

The 50 mg *M. uniflora* sample was weighed and placed into a 2 ml thick-walled centrifuge tube along with a 6 mm diameter steel ball. Next, 400 μl of 80% methanol solution (water/methanol = 1/4 (V / V)) was added. The frozen tissue grinder was used at 10°C and 50 Hz for 6 minutes. Then, the samples were subjected to low-temperature (5°C) ultrasonic extraction at a frequency of 40000 Hz for 30 minutes. Subsequently, the samples were left at 20°C for another 30 minutes, followed by centrifugation at 13 kg for 15 minutes at 4°C. The resulting supernatant was transferred to a vial for analysis. Each sample was mixed with 20 μl of the supernatant as a quality control sample.

2.5 Sample for quality control

The quality control (QC) sample is crucial to the experiment, serving to assess the stability of the analytical system during computer operation. This sample was a blend of all test samples (MuT1-6 + MuCK1-6), and was divided into six equal portions to obtain six QC samples for instrumental analysis. By assessing the repeatability of the QC samples, we can effectively evaluate the stability of the instrument throughout the entire analytical process. Furthermore, this approach allows us to pinpoint major variations in the analysis system, ensuring the reliability of the results.

2.6 UHPLC-MS/MS analysis

The metabolomic analysis was performed using a UHPLC-Q Exactive ultra-high performance liquid chromatography tandem Fourier transform mass spectrometry system (Waters Corporation, Milford, USA).

LC condition: 2 µl sample was separated on an HSS T3 column (100 mm × 2.1 mm i.d., 1.8 µm) and detected by mass spectrometry. The mobile phase A was 95% water + 5% acetonitrile (containing 0.1% formic acid). The mobile phase B was 47.5% acetonitrile + 47.5% isopropanol + 5% water (containing 0.1% formic acid). Separation gradient: 0–0.1 min, mobile phase B from linear 0% to 5%; 0.1–2 min, the mobile phase B increased from linear 5% to 25%; 2–9 min, the linearity of mobile phase B increased from 25% to 100%; 9–13 min, mobile phase B remains linear at 100%; and 13–13.1 min, the linearity of mobile phase B decreased from 100% to 0%. The linear range of mobile phase B was maintained at 0% within 13.1–16 min. Flowrate was 0.40 ml/min and column temperature were 40°C. The mass spectrometry data were collected using a Thermo UHPLC-Q Exactive HF-X mass spectrometer equipped with a positive and negative electrospray ionization (ESI) source. The optimal conditions were as follows: source temperature 425°C; sheath gas flow rate at 50 arb; auxiliary gas flow rate at 13 arb; ion-spray voltage floating (ISVF) at -3500 V in negative mode and 3500 V in positive mode; normalized collision energy at 20-40-60 V rolling for MS/MS. Full MS resolution was set to 60000, and MS/MS resolution was 7500. Data acquisition was performed with the Data Dependent Acquisition (DDA) mode. The sample mass spectrometry signal is acquired using positive and negative ion scanning modes with a mass scanning range of m/z 70 to 1050.

2.7 Data analysis

Statistical analysis of bioassay results was performed using SPSS version 23.0 (IBM-SPSS, Chicago, IL). Results of larval feeding consumption were analyzed using the independent samples Student's *t* test. Significant differences were determined by Tukey's least significant difference post hoc test ($P < 0.05$).

Progenesis QI (Waters Corporation, Milford, USA) was used to perform baseline filtering, peak identification, retention time correction, integration, and peak alignment on the raw data to obtain a data matrix of retention time, mass-to-charge ratio, and peak intensity. At the same time, MS and MS / MS mass spectrometry information were matched with the metabolic public database HMDB (<http://www.hmdb.ca/>) and Metlin (<https://metlin.scripps.edu/>) to obtain detailed information on metabolites.

The matrix data after searching the database is uploaded to the Meiji biological cloud platform (<https://cloud.majorbio.com>) for data analysis. In order to reduce the influence of variation in data-on-data analysis, to better screen, and to analyze differential metabolites, data preprocessing is first performed. The original data includes quality control (QC) samples and test samples. A series of preprocessing is required for the original data, including missing value filtering, missing value recoding, data normalization, QC verification and data conversion. Because the general RSD (relative standard deviation) greater than 30% of the variables fluctuates greatly during the experiment, it is necessary to eliminate the variables whose RSD exceeds the threshold, and to perform logarithmic processing to reduce or eliminate the asymmetry of the data structure and improve the normal distribution of the data structure. Then the data matrix can be obtained, and can be used for subsequent analysis.

The R software package *ropls* (version 1. 6. 2) was used for principal component analysis (PCA analysis), and seven cycles of cross-validation were used to evaluate the stability of the model. The selection of differential metabolites was based on the Variable Importance in Projection (VIP) obtained by the OPLS-DA model and the *P* value from student's *t* test. The metabolites with $VIP > 1$ and $P < 0.05$ were identified as differential metabolites. Differential metabolites were annotated by KEGG database (<https://www.kegg.jp/keg/pathway.html>). Pathway enrichment analysis was performed using the Python software package *scipy.stats*, and the biological pathways most relevant to the experimental treatment were obtained by Fisher's exact test.

3. Result

3.1 Effect of exogenous SA on growth and insect-resistance in *M. uniflora*

To quantify the effects of exogenous SA on *M. uniflora* growth and herbivory by *S. exigua*, stem diameter, plant height, and larval feeding consumption, were measured. The results showed that exogenous SA treatment had no significant effect on the stem diameter and plant height of *M. uniflora* ($P > 0.05$) (Fig. 1a-1b). The SA treatment did not cause damage to the plant. However, following exogenous SA induction, the feeding consumption of *S. exigua* larvae on *M. uniflora* was significantly reduced ($P < 0.05$) (Fig. 1c). For instance, a 61.1% reduction in feeding consumption was recorded on day 5 of the experiment, when compared with controls (68.1 ± 3.2 mg of control plants of *M. uniflora* sprayed with solvent (10% ethanol-water solution) vs 25.0 ± 0.5 mg of *M. uniflora* plants sprayed with 0.01 mol/l SA, $t = 4.329$, $df = 10$, $P < 0.001$).

3.2 Metabolite identification

The non-targeted metabolomic approach was employed to explore the exogenous SA-induced differences in metabolite profiles between the SA-treated plants of *M. uniflora* (MuT) and the control plants (MuCK). The 861 metabolites were identified under the mode of positive ions and 783 under negative ions (**Table S1-S2**). After preprocessing, 1,644 metabolites were identified in HMDB through secondary annotation. Subsequently, these metabolites were subsequently categorized into 13 superclasses, including 601 lipids and lipid-like molecules, 311 phenylpropanoids and polyketides, 207 organic oxygen compounds, 185 organoheterocyclic compounds, 127 organic acids and derivatives, 138 benzenoids, 20 nucleosides, nucleotides, and analogues, 16 alkaloids and derivatives, 18 lignans, neolignans and related compounds, 5 organic nitrogen compounds, 4 hydrocarbons, and 2 organosulfur compound hydrocarbon derivatives (Fig. 2). To gain a preliminary understanding of the differences in metabolite profiles between MuCK and MuT, Principal component analysis (PCA) was performed on the metabolic data matrices from both samples. According to the results from PCA analyses, exogenous SA induced significant alterations in the *M. uniflora* metabolome. The first two principal components accounted for 32.20% of the variance, and all samples were distributed within the 95% confidence

interval for outliers determined by Hotelling's T^2 analysis (Fig. 3a). A PLS-DA model was generated using 200 permutations, achieving a 95% confidence level and a score of 31.7% (Fig. 3b). In addition, high analytical reproducibility was indicated by the close clustering of all quality control (QC) samples with the six biological replicates from each sample in the principal component analysis (PCA). The first principal component accounted for 48.2% of the total variance, and the fifth principal component explained a further 99.5% (Fig. 3c). In PLS-DA replacement test, $R^2 = (0, 0.9239)$, $Q^2 = (0, -0.2634)$ (Fig. 3d). The significant differences in the metabolite profiles between MuCK and MuT indicate a substantial biological difference. The clustering heatmap analysis demonstrated statistically significant differences ($P < 0.05$) in the metabolite clustering patterns between MuCK and MuT. No statistically significant differences were observed within each group (Fig. 3e).

The obtained results demonstrated that the methods in the present study were significantly stable and reliable and could be used to yield high-quality metabolomic data suitable for subsequent analyses.

3.3 Orthogonal partial least squares-discrimination analysis

To better illustrate the differences between MuCK and MuT, we utilized OPLS-DA to emphasize the subtle variations. The results showed distinct clustering of MuCK and MuT samples, being consistent with the results from PCA, and indicated significant differences in metabolite profiles between the samples. OPLS-DA revealed the distinct differences in metabolic profile between MuCK and MuT, indicating the specific impact of exogenous SA on the metabolic profiles of *M. uniflora* (Fig. 4a). To prevent potential overfitting of the OPLS-DA model, 200 permutation tests were performed in this study. Theoretically, R^2 and Q^2 values close to 1 indicate high model accuracy. The simulated R^2 and Q^2 values are lower than those of the original model in the upper right corner, suggesting the original model's output is valid and reliable (Fig. 4b). The OPLS-DA model exhibited robust predictive capacity, thereby justifying its application in subsequent differential metabolite analysis.

3.4 Differential metabolomics analysis

To identify the key biochemical differences responsible for group separation, a loading plot of the OPLS-DA model was constructed. The load plot has a typical S-shape, representing the covariance and correlation between the metabolites and the model (Fig. 4c). The variables with higher p and p (corr) values are more important in distinguishing between two comparison groups. These variables are typically displayed in the upper right and lower left quadrants of the S plot, and variables with a Variable Importance in Projection (VIP) value greater than 1 are highlighted with red dots. The red dots represent related variables and are used to distinguish between the two groups.

A volcano plot is a useful method for visualizing data points with the highest degree of differential expression. It was used to further screen the differential metabolites between each two groups. It shows the relative concentration $\log_2$ fold change plotted against the P value versus the negative $\log_{10}$ fold change. The data points that are far from the rest in the graph show the metabolites with the most

variation. Significant differential metabolites were defined by a fold change ≥ 1 (upregulated) or ≤ 1 (downregulated), $P < 0.05$, and a VIP value ≥ 1 from OPLS-DA models. This analysis of 1644 metabolites revealed 321 upregulated and 137 downregulated metabolites in the MuCK vs MuT comparison (Fig. 4d). Based on fold change and VIP values, 30 metabolites were selected, including 24 upregulated metabolites and 6 downregulated metabolites (Fig. 5, **Table S3**).

3.5 Phytochemical classification analysis

According to the phytochemical classification, metabolites in plants can be categorized into primary and secondary metabolites. Primary metabolites are mainly involved in fundamental metabolic processes, such as respiration and energy production. In contrast, secondary metabolites often serve specialized functions, including defense against herbivores and pathogens, attracting pollinators, and allelopathy. According to a comparative analysis of 1,644 metabolites from MuCK and MuT, 435 primary metabolites and 809 secondary metabolites were identified, which included 5 primary categories and 12 secondary categories (Fig. 6a, Fig. 7a). In the 321 upregulated compounds, 79 primary metabolites and 157 secondary metabolites were identified, categorized into 5 primary categories and 11 secondary categories (Fig. 6b, Fig. 7b). Among the 137 downregulated compounds, 65 primary metabolites and 47 secondary metabolites were identified, also classified into 5 primary categories and 11 secondary categories (Fig. 6c, Fig. 7c). The 30 metabolites were selected based on differential fold change and VIP values, 11 primary metabolites and 14 secondary metabolites were identified, including 3 primary categories and 6 secondary categories (Fig. 6d, Fig. 7d).

The 79 upregulated and 47 downregulated compounds in exogenous SA-induced *M. uniflora* were categorized based on secondary metabolism, mainly including terpenes, phenolic acids and their derivatives, and flavonoids. Specifically, among terpenoids, carveol, farnesol, gibberellin A86, and gibberellin A7 were significantly upregulated, whereas linalool and β -ionone were found being significantly downregulated (Fig. 8, **Table S4**). Within phenolic acid and derivatives, the upregulated metabolites include SA, osmanthuside A, aspirin, gallic acid, 4-hydroxybenzoic acid, benzoic acid, and caffeic acid. Conversely, the downregulated metabolites include 2-pyrocatechuic acid and (*E*)-1-cinnamoylpyrrolidine (Fig. 9, **Table S5**). Regarding flavonoid metabolites, the upregulated metabolites consist of eupatilin, peonidin 3-rutinoside, and 4', 5-dihydroxy-7, 8-dimethoxyflavone. The downregulated flavonoid metabolites include malvin, and peonidin 3-rhamnoside 5-glucoside (Fig. 10, **Table S6**).

3.6 Metabolic pathway enrichment analysis

The KEGG database was employed for pathway enrichment analysis to elucidate alterations of metabolic pathways between MuCK and MuT. Comparisons between MuCK and MuT revealed that differentially accumulated metabolites (DAMs) were predominantly enriched in 100 specific pathways or metabolic processes (**Table S7**). The KEGG enrichment histogram highlights the top 20 enriched pathways across the different samples. The pathways that were significantly enriched included those related to plant hormone signaling, EIP (early induced pathway), linoleic acid metabolism, cofactor

biosynthesis, ABC transporters, the biosynthesis of various plant secondary metabolites, arachidonic acid metabolism, tyrosine metabolism, phenylalanine metabolism, and nucleotide metabolism (Fig. 11(a)). Key enrichment pathways among the 321 up-regulated metabolites included phenylpropanoid biosynthesis, α -linolenic acid metabolism, and the production of diverse plant secondary metabolites, α -linolenic acid metabolism, and the biosynthesis of various secondary metabolites in plants (Fig. 11(b)). In contrast, enrichment analysis indicated that the 137 downregulated metabolites were significantly enriched in pathways associated with nucleotide metabolism, cyanoamino acid metabolism, and arginine biosynthesis (Fig. 11(c)). The upregulated and downregulated terpenoids, phenolic acids and their derivatives, as well as flavonoids, were enriched in the metabolic pathways, including *N*-glycan biosynthesis, plant hormone signal transduction, biosynthesis of various plant secondary metabolites, monoterpene biosynthesis, and limonene and pinene degradation pathways. These pathways play critical roles in enhancing the resistance of *M. uniflora* following induction by exogenous SA (Fig. 11(d)).

4. Discussion

This study uses metabolomics to elucidate the mechanisms underlying the enhanced disease and pest resistance in *M. uniflora* induced by exogenous SA, focusing on characterizing changes in the metabolome and identifying key metabolites involved.

4.1 The global metabolomic response to exogenous SA induction

In this study, we verified that the resistance in plants against insects can be induced by SA (Fig. 1c) through alteration of plant secondary metabolites. Generally, SA pathway in plants is mainly involved in resistances of plants against phytopathogens (Wang et al., 2009; Wang et al., 2020). However, many researches also demonstrated that SA can reliably induce the plant resistances to herbivorous insects by enhancing systemic acquired resistance (SAR) in plants (Vlot et al., 2009; Yang et al., 2015). For example, the obvious resistance in rose plants against *S. exigua* can be induced by exogenous SA, being similar to the induction by infection of roses with *Podosphaera pannosa* (Yang et al., 2022).

From the perspective of changes in secondary metabolites, our findings demonstrate that terpenoids, phenolic acids and their derivatives, and flavonoids in *M. uniflora* play critical roles in mediating the enhanced resistance induced by exogenous SA. The diverse array of terpene secondary metabolites plays a critical role in plant defense, which are involved in multiple mechanisms against both insect herbivores and plant pathogens, such as direct toxicity, feeding deterrence, and immune system modulation, alongside the attraction of beneficial natural enemies (Maharajya et al., 2019; Pingault et al., 2021; Wang et al., 2021; Li et al., 2023). Phenolic acids and their derivatives are also crucial for plant defense, exhibiting both direct antimicrobial effects against pathogens and indirect effects such as enhanced immunity and herbivore resistance through repellent or toxic properties (Magalhães et al., 2010; Szmiański et al., 2015; Oszmiański et al., 2015; Florencio-Ortiz et al., 2021). Flavonoids can

function as signaling molecules in plants, playing a crucial role in mediating defense responses, thereby enhancing plant resistance to diseases and pests (Yan et al., 2015; Desmedt et al., 2021; Ramaroson et al., 2022; Wang et al., 2024). This study showed that exogenous SA induced significantly the greater changes in terpenoids, phenolic acids and their derivatives, and flavonoids in *M. uniflora* than in other secondary metabolite classes, highlighting the importance of these compounds in mediating enhanced resistance to herbivores and pathogens (Fig. 7). In agreement with our findings, exogenous SA treatment significantly increased terpenoid content in *Sanguangporus baumii* (Wang et al., 2023). The enhanced levels of flavonoids and phenolic compounds in *Panax vietnamensis* following exogenous SA treatment suggest a potential mechanism for improved stress resistance (Gondor et al., 2016; Hou et al., 2024).

4.2. Roles of terpenoids on the resistance of *M. uniflora*

Our results revealed that terpenoids were the major contributors to the observed metabolic differences between exogenous SA-treated and control *M. uniflora* plants (Figs. 7, 8). The previous study also demonstrated that exogenous SA can induce the activation of plant signaling pathways, leading to increased biosynthesis of many secondary metabolites, including gibberellic acid (GA3, a diterpenoid), azelaic acid, and jasmonic acid, thereby enhancing plant defenses. Subsequently, these defensive responses reduce fitness of *Spodoptera frugiperda* and suppress its feeding (Gordy et al., 2015; Alvarenga et al., 2017). Gibberellic acid application effectively reduced chlorosis and *Melanocallis caryaefoliae* populations on pecan leaves, particularly mitigating pest outbreaks following broad-spectrum insecticide use (Cottrell and Burrack, 2022). In addition to SA, exogenous methyl salicylate (MeSA) application can also increase resistances of plants. For instance, the release of specific volatile organic compounds (VOCs) from wheat plants, including carveol (a monoterpene), can be induced by MeSA, thereby attracting the parasitoid *Aphidius gifuensis* and mediating indirect defense against aphids (Xiao et al., 2022). Studies have shown that the presence of farnesol in essential oils is correlated with insecticidal efficacy against *Cerosipha forbesi* infesting strawberry crops (Bravim dos Santos et al., 2021). Furthermore, the release of a blend of mono- and sesqui-terpenes from maize (*Zea mays*) can be triggered by infestation by the Egyptian cotton leafworm (*Spodoptera littoralis*), thus attracting parasitoid wasps for indirect defense. Additionally, Schnee et al. (2002) cloned a maize terpene synthase gene, *tps1*, encoding the enzyme that produces (*E*)-farnesene and (3*E*)-4, 8-dimethyl-1, 3, 7-nonatriene (major components of the herbivore-induced VOCs blend). The expression of *tps1* is regulated by herbivory, mechanical wounding, and elicitor treatments. Linalool acts as an attractant for various phytophagous insects, including *S. exigua*, *Spodoptera frugiperda*, *Epilachna vigintioctopunctata*, and *Aulacophora foveicollis*, facilitating indirect defense mechanisms in plants (Sarkar et al., 2016; Yang et al., 2022; Liu et al., 2023). β -Ionone demonstrates significant attractant effects on whiteflies, *S. exigua*, and *Lasioderma serricorne* (Phoonan et al., 2014; Liu et al., 2017; Qi et al., 2024). Thus, we hypothesize that terpenoids can play an important role in the enhanced resistance of *M. uniflora* against *S. exigua*. In the present study, exogenous SA resulted in the upregulation of gibberellin A86 and gibberellin A7 and the downregulation of β -ionone, linalool in *M. uniflora*. These significant changes in terpenoids may primarily be responsible for the increased resistance in *M. uniflora* against *S. exigua*. These findings largely

elucidated the chemical defense mechanisms underlying how the resistance in *M. uniflora* to combat *S. exigua* infestation was induced by exogenous SA.

4.3. Effects of phenolic acids and their derivatives

The effect of exogenous SA treatment on *M. uniflora* was assessed by analyzing changes in the relative content of phenolic acids and their derivatives. Statistically significant differences were observed in MuT and MuCK (Figs. 7, 9). Exogenous SA triggers a cascade of events including the activation of benzoic acid and aspirin biosynthesis pathways, along with the upregulation of endogenous SA-mediated defense signaling. This cascade results in the increased production of defense-related metabolites, thereby enhancing plant resistance to pathogens (Qi et al., 2012; Mukherjee et al., 2012; Killiny and Nehela, 2017; Lyousfi et al., 2021; Abo-Elyousr et al., 2022). Caffeic acid plays a crucial role in tobacco resistance to *Ralstonia solanacearum*, with concentrations significantly elevated to 1.95 mg/μl in infected plants, directly disrupting bacterial cell membranes and inhibiting biofilm formation (Li et al., 2021). Gallic acid and caffeic acid exhibit significant protective effects against *Botrytis cinerea* in strawberries (Bilgin et al., 2021). The study demonstrated that silicon application significantly increases the levels of gallic acid and its derivatives in chestnut plants, leading to enhanced resistance against *chestnut blight* (Carneiro-Carvalho et al., 2019). The enhanced disease and insect resistance in SA-treated *M. uniflora* may be mediated by the upregulation of specific defense pathways. Many studies showed that SA can enhance the resistances of plants against insects (Zhang et al., 2019; Han et al., 2025; Liu et al., 2025). Specifically, increased levels of benzoic acid, SA, and aspirin potentially contribute to enhanced disease resistance, while gallic acid and caffeic acid may play a role in strengthening insect resistance. The increase in SA content in *M. uniflora* induced by exogenous SA can be attributed to three potential mechanisms, including direct incorporation of the exogenous compound, induction of endogenous SA biosynthesis, or a combination of these processes.

4.4 Effects of flavonoids

Our results showed that the contents of flavonoids in *M. uniflora* were changed after exogenous SA treatment (Figs. 7, 10). The enhanced pest resistances in *M. uniflora* should be also associated with the changes in the flavonoids. The previous researches also elucidated that insect resistances in plants were related to the levels of flavonoids. For instance, the *R1* locus, harboring the MYB transcription factor GhPAP1D, regulates anthocyanin accumulation and enhances insect resistance in cotton. GhPAP1D overexpression increases anthocyanin levels and improves resistance to cotton bollworm and spider mites in transgenic tobacco and cotton (Li et al., 2019). Strigolactone (SL) signaling regulates defense responses in *Nicotiana attenuata* against *Trichobaris mucorea* feeding at the root-stem junction. Silencing of SL pathway genes (NaMAX2, NaD14, NaCCD7) resulted in increased anthocyanin and phenolamide accumulation in stems, enhancing resistance to *T. mucorea* (Estelle et al., 2020). In our research, enhanced disease and insect resistance in *M. uniflora* following exogenous SA treatment may be attributed to the significant upregulation of the flavonoids such as peonidin 3-rutinoside and the significant downregulation of the flavonoids such as malvidin and peonidin 3-rhamnoside 5-glucoside.

5. Conclusions

Exogenous SA is widely studied for its roles in enhancing plant resistance to diseases and herbivorous insects. This study analyzed the metabolomic changes and herbivorous resistance in *M. uniflora* induced by exogenous SA. Results showed that exogenous SA had no significant effect on the growth of *M. uniflora*, including stem diameter and plant height. However, it significantly enhanced resistance of *M. uniflora* to *S. exigua* larvae. Metabolomic analysis revealed substantial changes in secondary metabolites, with 79 upregulated and 47 downregulated compounds. The major metabolite classes were terpenoids, phenolic acids and their derivatives, and flavonoids, which most likely contributed to the significantly enhanced resistance. Among terpenoids, carveol, farnesol, gibberellin A86, and gibberellin A7 were upregulated, while linalool and β -ionone were downregulated. For phenolic acids and derivatives, upregulated compounds included benzoic acid, 4-hydroxybenzoic acid, gallic acid, SA, aspirin, and caffeic acid, whereas downregulated compounds included 2-pyrocatechuic acid and (*E*)-1-cinnamoylpyrrolidine. Notable changes in flavonoids included the upregulation of 4', 5-dihydroxy-7, 8-dimethoxyflavone and the downregulation of malvidin and peonidin 3-rhamnoside 5-glucoside. Several metabolic pathways were involved in the synthesis of these metabolites, including phenylpropanoid biosynthesis, monoterpene biosynthesis, limonene and pinene degradation, and phenylalanine metabolism. These pathways played critical roles in strengthening the insect resistance and potential defense mechanisms of *M. uniflora* following exogenous SA treatment. Our study may provide a potential approach and insight for the prevention and control of pests and diseases in *M. uniflora*.

Declarations

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Ethics approval and consent to participate

Not applicable.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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Figures

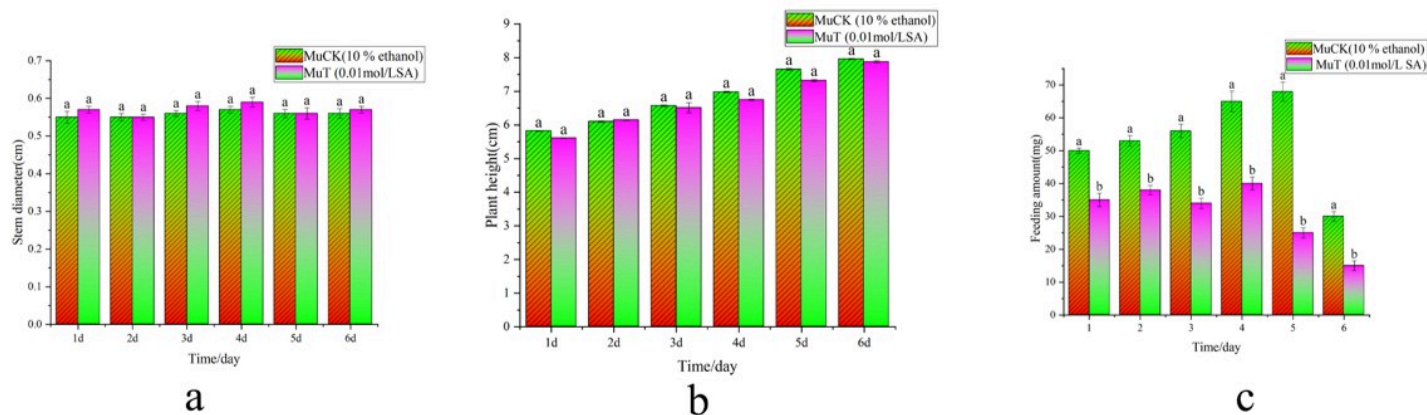


Figure 1

Effects of exogenous SA on changes in stem diameter, plant height of *M. uniflora*, and tissue amount consumed by *S. exigua* on *M. uniflora*. (a) Stem diameter of *M. uniflora*. (b) Plant height of *M. uniflora*. (c) Feeding amount of *S. exigua* on *M. uniflora*. For each experiment, ten plants of *M. uniflora* were sprayed with 0.01 mol/l SA in 10% (V/V) ethanol-water (MuT), while another ten plants of *M. uniflora* were treated with 10% (V/V) ethanol-water was used as control (MuCK). Different lowercase letters indicate significant differences ($P < 0.05$).

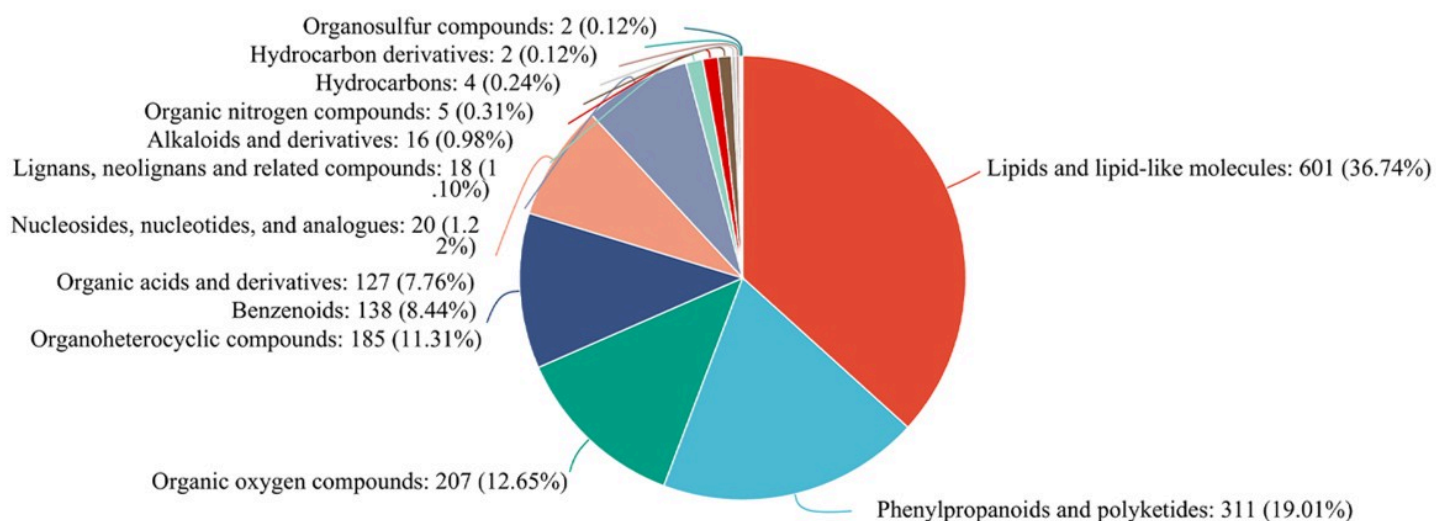


Figure 2

Classification of the identified metabolites annotated using the HMDB database.

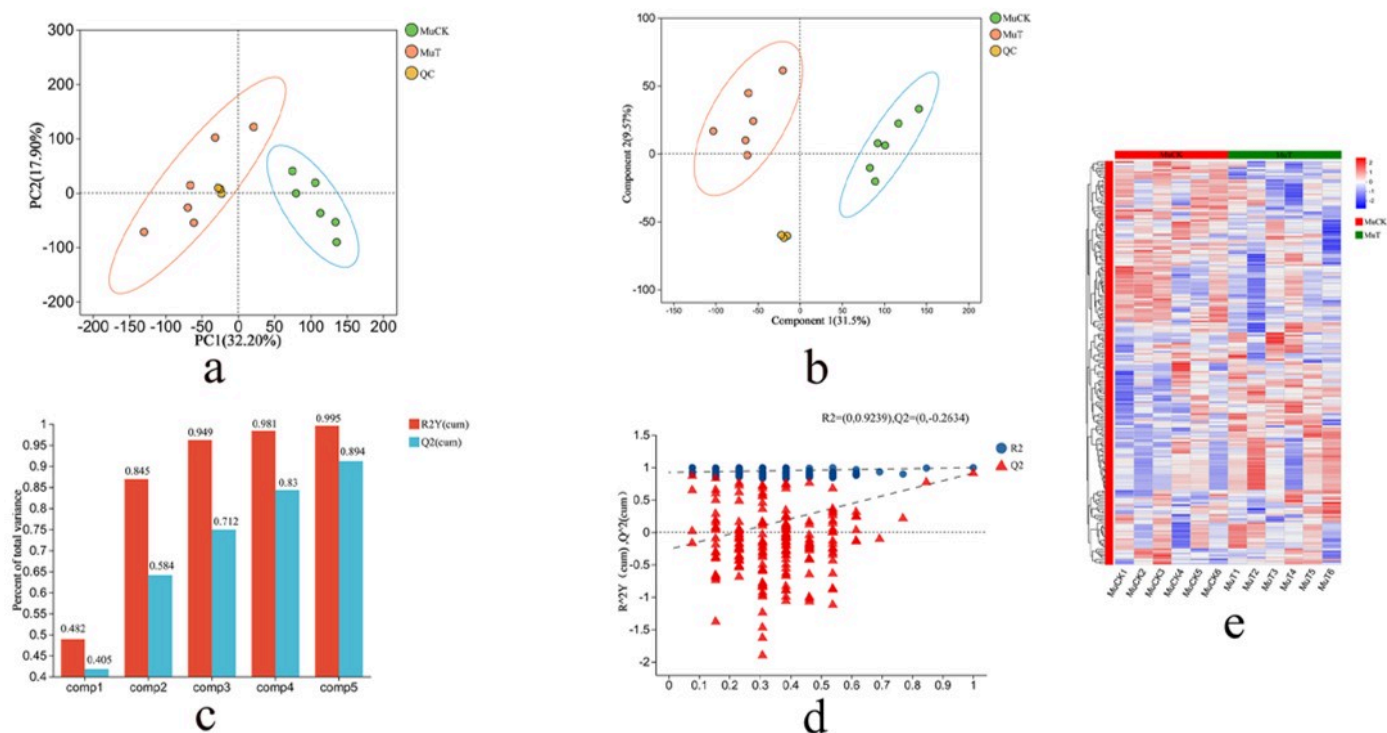


Figure 3

Principal component analysis (PCA), Score plot, Model overview and permutation test of Partial Least Squares Discriminant Analysis (PLS-DA) of the metabolite's distribution between MuT and MuCK in *M. uniflora*. (a) PCA was employed to compare the metabolomic profiles of two sample groups. (b) Score (PLS-DA) plot under the comparison of two groups of samples. (c) PLS-DA Model overview for comparison of two groups of samples. (d) PLS-DA permutation test under the comparison of two groups of samples. (e) Hierarchical cluster analysis (HCA) of metabolites.

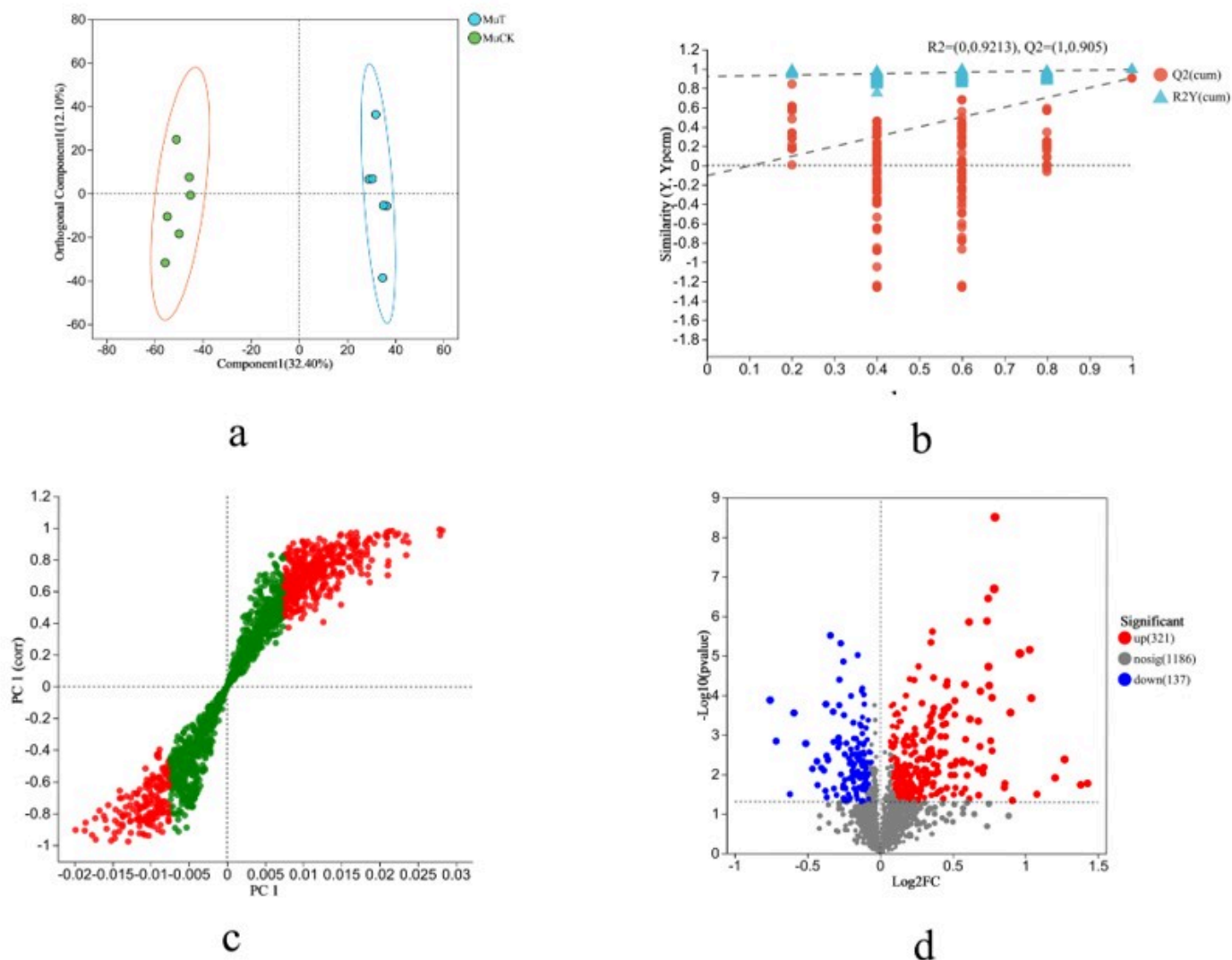


Figure 4

Differential metabolite analysis for the comparison between MuT and MuCK using orthogonal partial least squares-discrimination analysis (OPLS-DA). (a) Scores (OPLS-DA) plot in differential metabolite analysis. (b) OPLS-DA permutation test in differential metabolite analysis. (c) Corresponding OPLS-DA loading S-plot, the abscissa represents the predicted principal component, and the ordinate is the orthogonal principal components, the variables with VIP > 1.0 were highlighted with red. (d) Volcano plot. Red dots represent up-regulated metabolites, blue dots represent down-regulated and gray is insignificant difference metabolites.

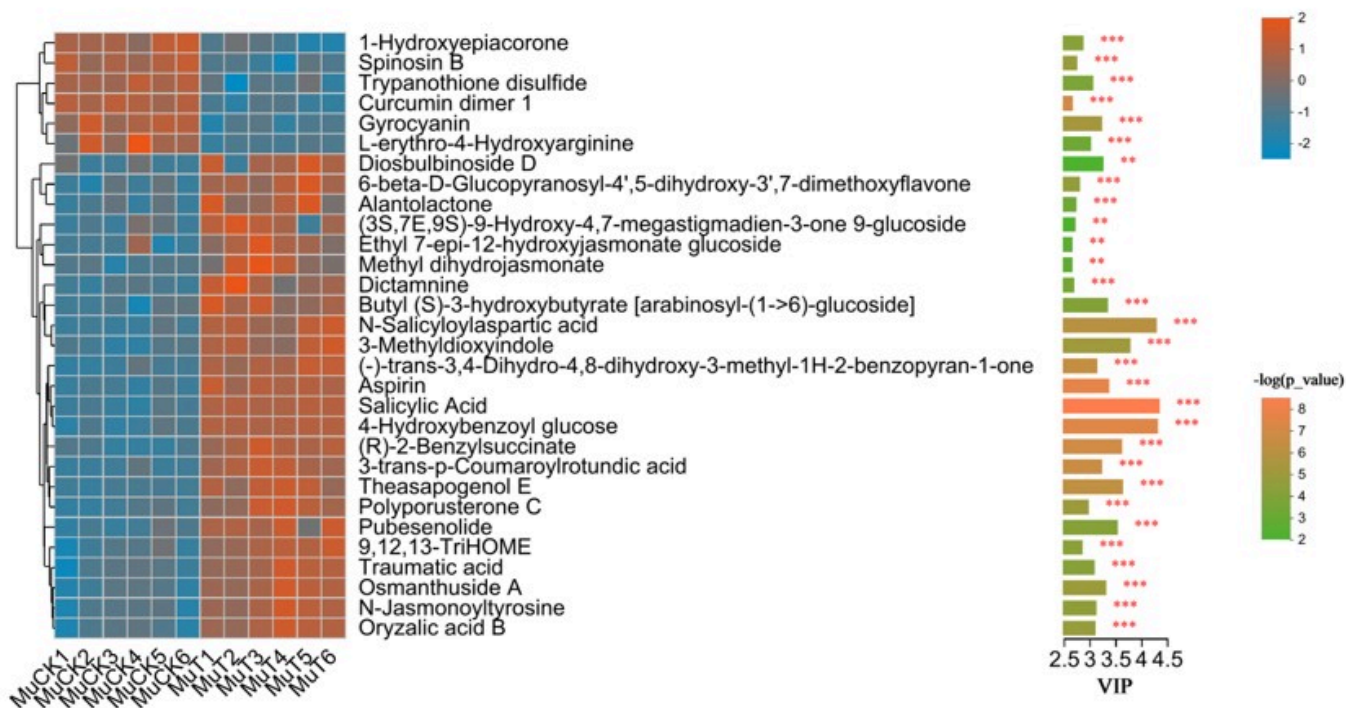


Figure 5

VIP values and fold changes of metabolites were analyzed. The left side is the metabolite clustering tree. The metabolite VIP bar chart is on the right side. The bar color indicates the significant difference in metabolites between the two groups of samples, i.e., P_value. * represents $P < 0.05$, ** represents $P < 0.01$, and *** represents $P < 0.001$.

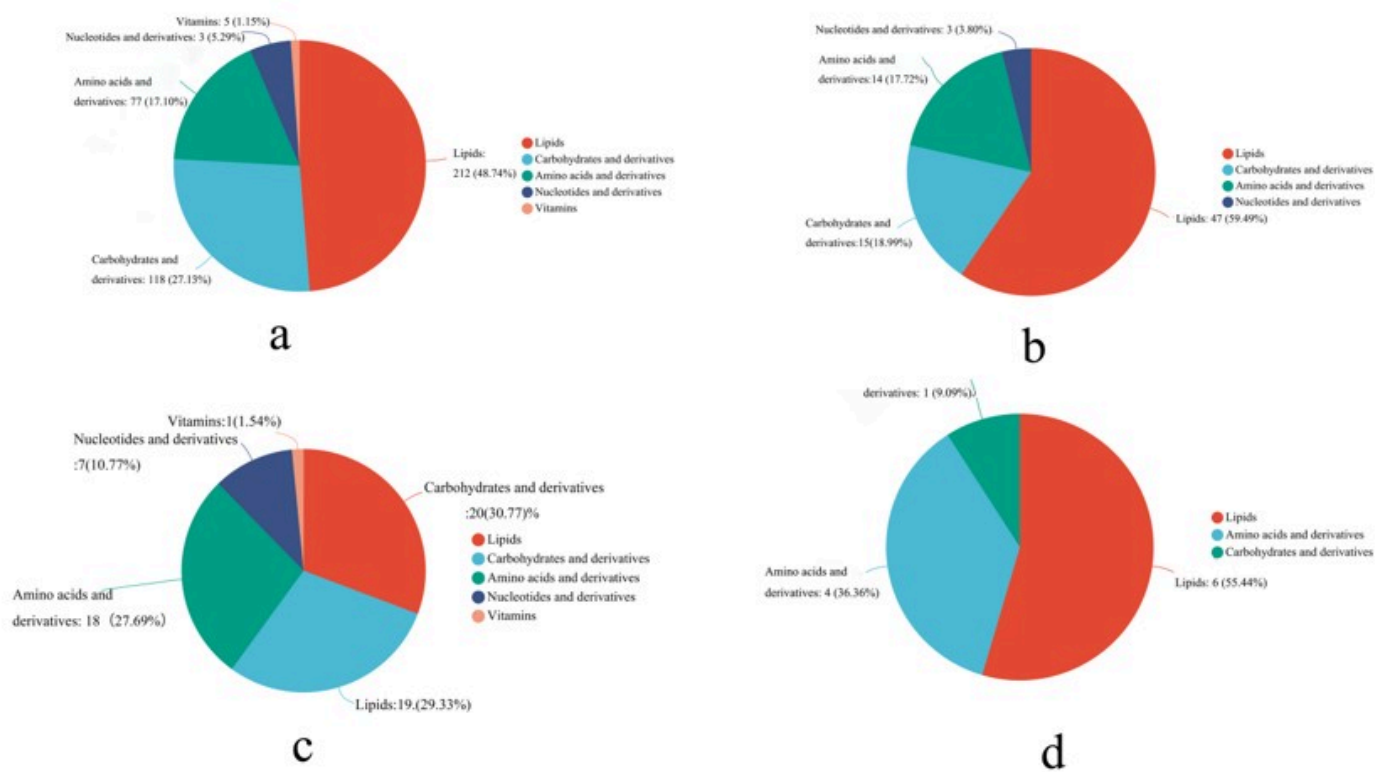


Figure 6

Classification of the identified metabolites annotated using the primary method in phytochemistry. (a) Classification of the identified 435 metabolites annotated using the primary method. (b) Classification of the identified 83 up-regulated metabolites annotated using the primary method. (c) Classification of the identified 65 down-regulated metabolites annotated using the primary method. (d) 11 metabolites, selected based on VIP values and fold change were classified using the primary method.

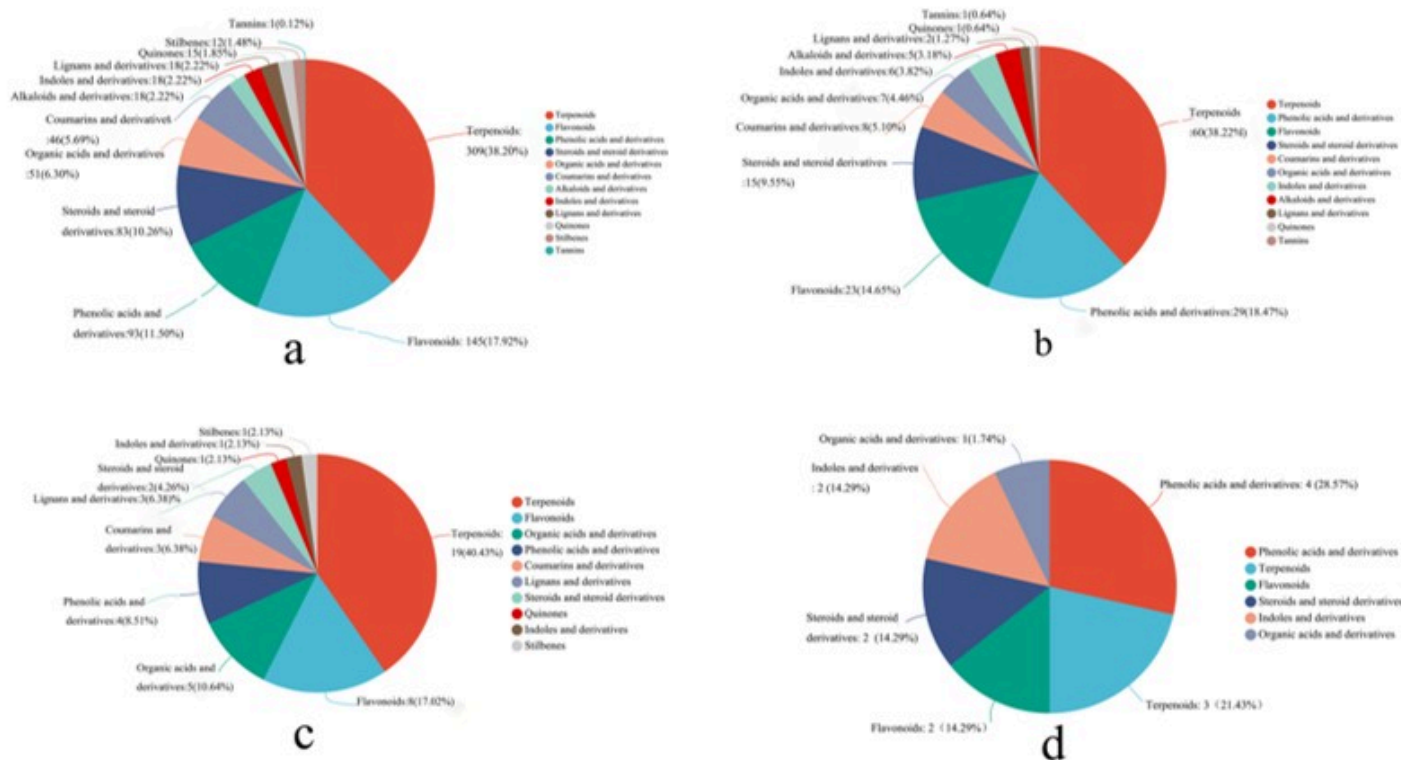


Figure 7

Classification of the identified metabolites annotated using the secondary method in phytochemistry. (a) Classification of the identified 809 metabolites annotated using the secondary method. (b) Classification of the identified 157 up-regulated metabolites annotated using the secondary method. (c) Classification of the identified 47 down-regulated metabolites annotated using the secondary method. (d) 14 metabolites, selected based on VIP values and fold change, were classified using the secondary method.

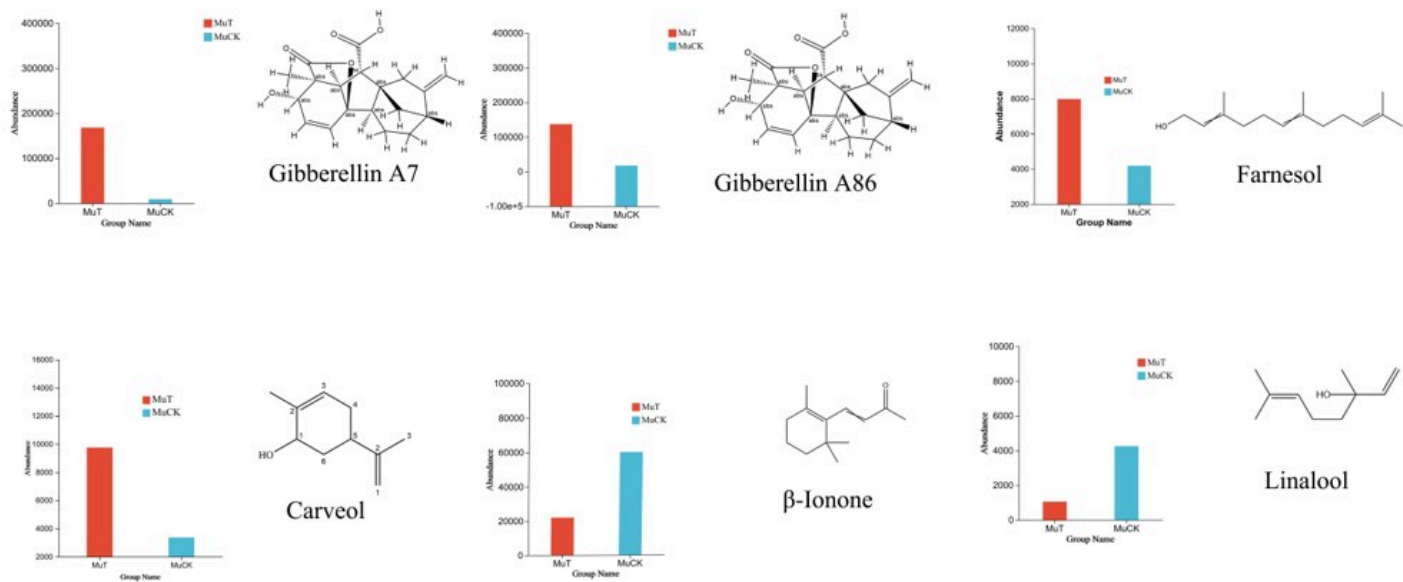


Figure 8

Some upregulated and downregulated terpenoids.

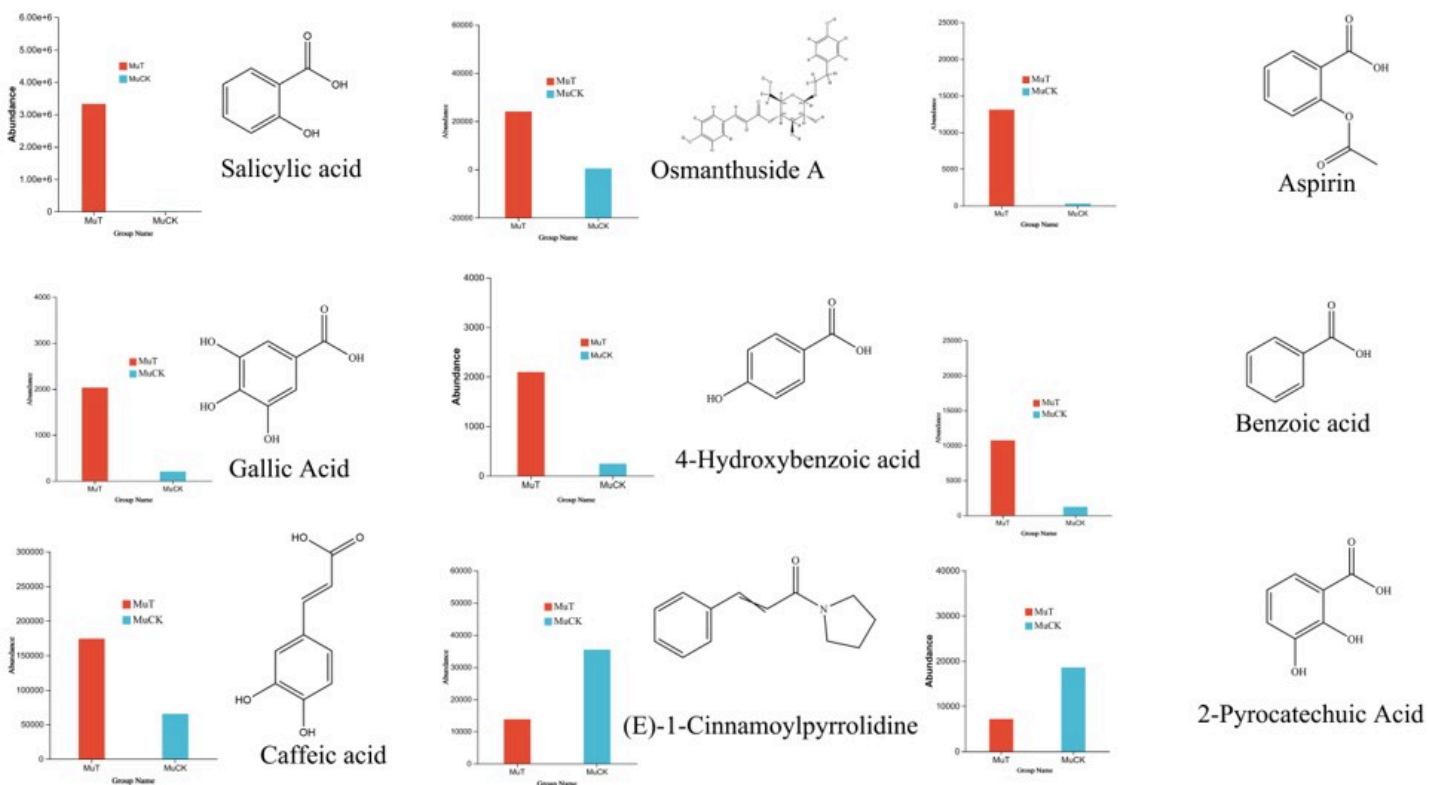


Figure 9

Some upregulated and downregulated phenolic acids and derivatives.

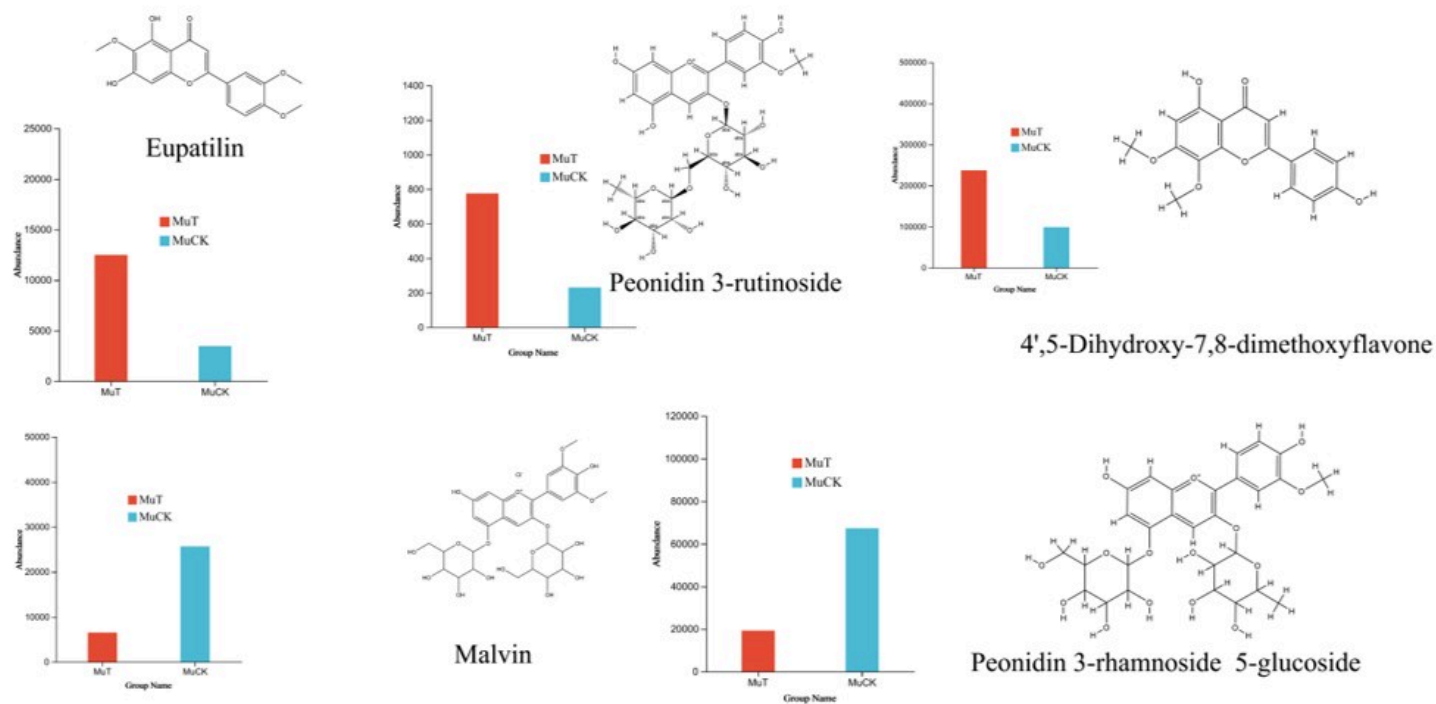


Figure 10

Major flavonoids with upregulated and downregulated expressions.

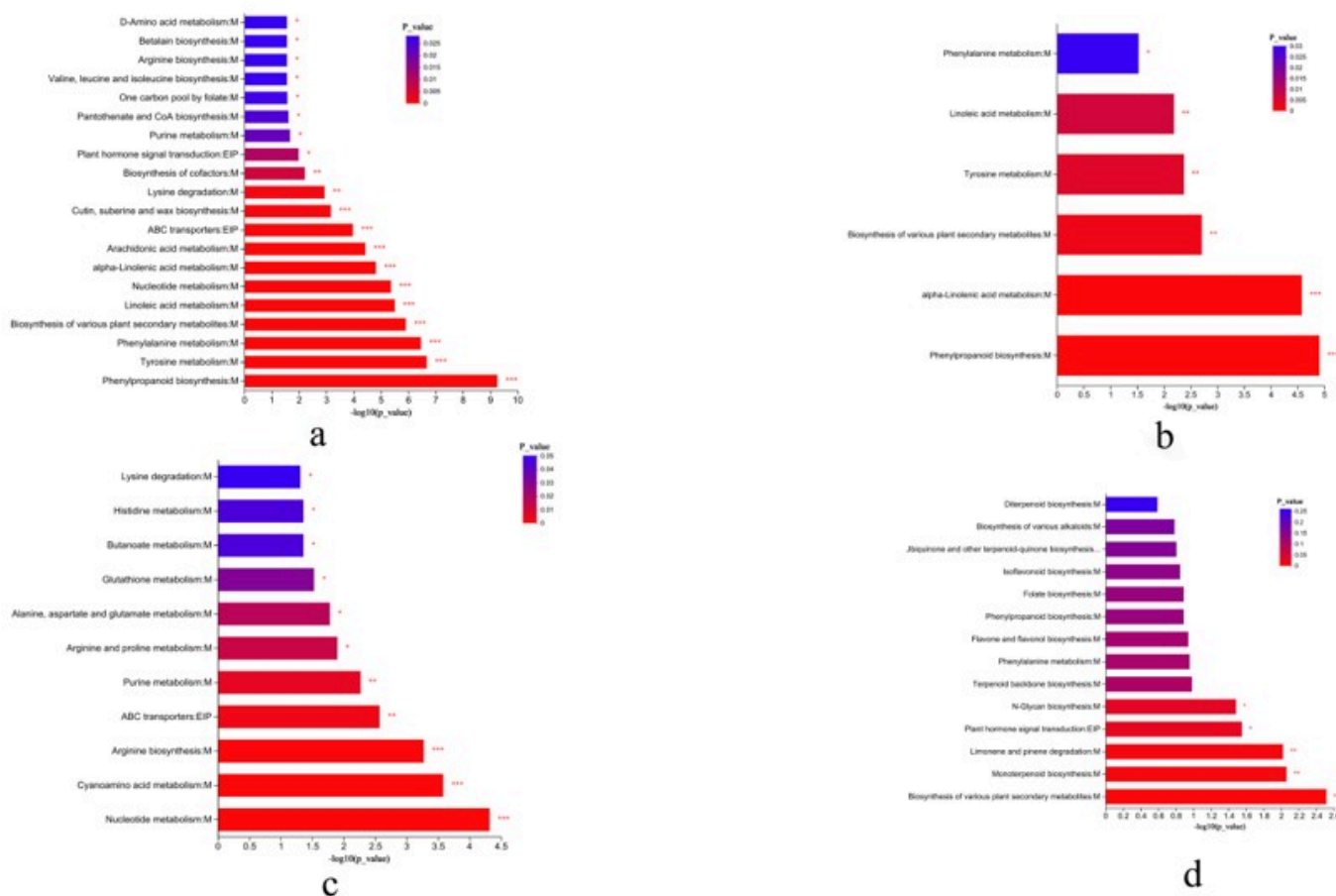


Figure 11

KEGG pathways of the differentially accumulated metabolites (DAMs) by comparing between MuT and MuCK. (a) The top 20 enriched KEGG pathway of 458 DAMs. (b) KEGG pathways of 321 upregulated metabolites. (c) KEGG pathways of 137 downregulated metabolites. (d) The key KEGG pathways with critical roles in enhancing the resistance of *M. uniflora*. *** represents $P < 0.001$, ** represents $P < 0.01$, and * represents $P < 0.05$.

Supplementary Files

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

- [TableS1OverviewformetabolitesinMonotropauniflora.xlsx](#)
- [TableS2TotalNumbersofmetaboliteidentified.xlsx](#)
- [TableS3VIPvalueof30compounds.xlsx](#)
- [TableS4terpenoids.xlsx](#)
- [TableS5phenolicacidandderivatives.xlsx](#)
- [TableS6flavonoids.xlsx](#)

- [TableS7KEGGpathwayenrichment.xlsx](#)