

Multionomics Reveals Mechanisms of *Alternaria oxytropis* Inhibiting Pathogenic Fungi in *Oxytropis ochrocephala*

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ABSTRACT: Endophytic fungi can benefit the host plant and increase the plant resistance. Now, there is no in-depth study of how *Alternaria oxytropis* (*A. oxytropis*) is enhancing the ability of inhibiting pathogenic fungi in *Oxytropis ochrocephala* (*O. ochrocephala*). In this study, the fungal community and metabolites associated with endophyte-infected (EI) and endophyte-free (EF) *O. ochrocephala* were compared by multionomics. The fungal community indicated that there was more *A. oxytropis*, less phylum Ascomycota, and less genera *Leptosphaeria*, *Colletotrichum*, and *Comoclathris* in the EI group. As metabolic biomarkers, the levels of swainsonine and apigenin-7-O-glucoside-4-O-rutinoside were significantly increased in the EI group. Through in vitro validation experiments, swainsonine and apigenin-7-O-glucoside-4-O-rutinoside can dramatically suppress the growth of pathogenic fungi *Leptosphaeria sclerotoides* and *Colletotrichum americanae-borealis* by increasing the level of oxidative stress. This work suggested that *O. ochrocephala* containing *A. oxytropis* could increase the resistance to fungal diseases by markedly enhancing the content of metabolites inhibiting pathogenic fungi.

KEYWORDS: *Oxytropis ochrocephala*, *Alternaria oxytropis*, swainsonine, pseudotargeted metabolomics, high-throughput sequencing of ITS

INTRODUCTION

Locoweed, a perennial herbaceous plant that is sometimes associated with severe toxicity to grazing livestock, is common in pastures and ranges of arid and semiarid regions of China and the United States.¹ *Oxytropis ochrocephala* (*O. ochrocephala*), a major locoweed in China, can host an endophytic fungus *Alternaria oxytropis* (*A. oxytropis*), which produces the toxin swainsonine.² Livestock that consume plants containing swainsonine can suffer from neurological disorders, such as wobbly gait and muscle incoordination,³ thus inducing losses in the livestock industry. In addition, damage in other grassland plants due to salinization and rodents have aided in the growth of *O. ochrocephala*. Consequently, *O. ochrocephala* has become a dominant plant, thereby affecting the development of the ecological environment.⁴ *O. ochrocephala* is a high-quality legume pasture with high nutritional value, but it is not widely planted and utilized at present because it is a noxious locoweed. After adding silage to the diet to feed the test sheep, not only the test sheep did not have the poisoning phenomenon of locoweed but also the addition of silage to the diet had better palatability and did not affect the intake of the test sheep.⁵ If ecologically adaptive locoweed can be utilized, the shortage of agricultural herbage may be alleviated. This study can provide a basis for the agricultural resource utilization of *O. ochrocephala*.

Endophytic fungi grow inside host plants without causing symptoms of pathogens. Many endophytic fungi are thought to confer some benefit to the host, including enhancing the host plant's growth and improving the plant's adaptability and microbiome diversity.⁶ For instance, *Paraconiothyrium estuar-*

inum, an endophytic fungus associated with *Brachiaria brizantha*, has been found to elevate drought tolerance and sustainable growth ability of host plants.⁷ Endophytic fungi can resist biotic stressors by secreting metabolites such as antibiotics and activating plant defenses.

Despite their benefits, an undesirable characteristic of many endophytes is that some of their secondary metabolites may have toxic effects on livestock. For example, certain ergot alkaloids and indole diterpene alkaloids generated by some endophytic *Epichloë* can be poisonous to livestock.⁸ *A. oxytropis*, an endophyte of locoweed such as *O. ochrocephala*, can produce swainsonine, which can inhibit the activity of α -mannosidase II and lead to symptoms of toxicity in livestock.⁹

The risk of plant diseases caused by fungal pathogens has significantly increased in recent years, posing a serious threat to the plant ecology, food production, and human health. Anthracnose, caused by the pathogenic fungus *Colletotrichum*, is one of the major constraints for soybean production.¹⁰ *Leptosphaeria sclerotoides* is a common pathogenic fungus of alfalfa.¹¹ *Comoclathris* may play an essential role as a pathogenic fungus in causing *Bromus tectorum* stand failure.¹²

In recent years, integrative analysis of microbiomes and metabolomes has become increasingly extensive in studies of

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human health, plant disease resistance, and nutrition of cash crops.¹³ Previous studies confirmed that *Epichloë* species can enhance salt tolerance by improving metabolite contents¹⁴ of *Achnatherum inebrians*. Kou et al.¹⁵ found that *Epichloë* could increase the levels of resistance to a pathogen by regulating the salicylic acid signaling pathway in *A. inebrians*. For integrated microbiome and metabolome analysis, experiments have associated the presence of *Epichloë* with increased micro-organism α -diversity and several metabolites.¹⁶ To date, similar studies investigating the microbiome and metabolome of *O. ochrocephala* are lacking. Based on the fungal community and metabolism of *O. ochrocephala* plants infected and not infected with the symbiont *A. oxytropis*, the objective of this study was to reveal the internal mechanism by which endophytic fungi enhance host disease resistance.

MATERIALS AND METHODS

Sample Collection and Treatment. *O. ochrocephala* stems and leaves at the flowering stage were collected from a natural grassland located at Xiahe County, Gannan Tibetan Autonomous Prefecture, Gansu Province (102°27'E, 35°3'N, altitude 3171 m) in July 2021. All of the plants collected looked healthy and did not contain obvious signs or symptoms of pathogenic fungi. Totally, 60 samples were collected, and the stems and leaves were cleaned by ultrapure water. Then, each individual sample was aliquoted into two sterile centrifuge tubes. The stems and leaves in one tube for fungal community sequencing and detection of metabolites; the aliquot was immediately frozen in liquid nitrogen and preserved at -80°C . The aliquot in the other tube was used for molecular identification of endophytic fungi and subsequent experiments. A flowchart of the experimental setup is shown in Figure S1.

Microscopic Observation and Molecular Identification of Endophytic Fungi from *O. Ochrocephala*. In the laboratory, part of each sample was washed under tap water for 5 min; the soil, miscellaneous bacteria, and other substances on the surface of samples were washed away; and then disinfected in 75% ethanol for 1–2 min and 1% sodium hypochlorite solution for 2–3 min, washed with sterile water, and dried with sterile filter paper. The stem bases of 60 samples stained with aniline blue were examined under a microscope to determine the presence or absence of endophytic fungi. The stem of each *O. ochrocephala* was cut from the cross section and stained with aniline blue. A small amount of *O. ochrocephala* stem pulp was scraped with a scalpel and coated on the slide (three times for each piece). A few drops of aniline blue dye solution were dripped on the samples on the slide, and then, the slide was put under the ordinary microscope for observation.¹⁷

We extracted DNA from 100 mg of stem of 60 plant samples using an Ezup Spin Column Super Plant DNA Kit (Sangon Biotech Co., Ltd., Shanghai, China). The samples were pulverized to powder with a Qiagen TissueLyser (Retsch GmbH Co., Ltd., Hannover, Germany). The T100 Thermal Cycle PCR (Bio-Rad Laboratories Co., Ltd., Shanghai, China) was used to amplify fungal target genes. PCRs were conducted in a 25 μL solution (contained 1 μL of each primer, 1 μL of genomic DNA, 9.5 μL of ultrapure water, and 12.5 μL of 2X SanTaq PCR Master Mix) with the following cycling conditions: 95 $^{\circ}\text{C}$ for 10 min, 32 cycles (94 $^{\circ}\text{C}$ for 45 s, 52 $^{\circ}\text{C}$ for 45 s, and 72 $^{\circ}\text{C}$ for 45 s), and 72 $^{\circ}\text{C}$ for 10 min. The PCR primers are listed in Table S1. PowerPac Basic Electrophoresis Apparatus (Bio-Rad Laboratories Co., Ltd., Shanghai, China) was used to detect PCR products.

For molecular identification, samples with bright and clear Mtssu gene bands were identified as positive for *A. oxytropis*. Samples with dark Mtssu gene bands were negative for *A. oxytropis*. For microscopic observation, samples with stained mycelium were identified as positive for *A. oxytropis*, and samples without stained mycelium were negative for *A. oxytropis*. Plants both positive in microscopy and molecular identification were selected as endophyte-infected (EI) and the contrary for endophyte-free (EF).

High-Throughput Sequencing of Ribosomal Internal Transcribed Spacer (ITS). Total DNA of 100 mg of plant samples was extracted using the DNA Kit (Biogothie Biotechnology Co., Ltd., Wuhan, China). The specific PCR primers used for *Mtssu* gene and ITS are shown in Table S1. *Mtssu* genes were specific to detect the presence of *A. oxytropis* in the plant tissue,¹⁸ and ITS primers were for detecting the presence of other fungi including *A. oxytropis*. The specific sequencing methods are placed in Methods S1 of the Supporting Information.

Pseudotargeted Metabolomics Analysis. Pseudotargeted metabolomics merged the advantages of untargeted and targeted metabolomics with outstanding sensitivity and specificity and superior quantitative capabilities. Pseudotargeted metabolomics analysis including sample extraction and LC-ESI-MS/MS analysis is shown in Methods S2 of the Supporting Information.

Metabolomics Data Analysis, Fungal Microbiome Data Analysis, and Statistical Analysis. We obtained metabolic profiling based on LC-ESI-MS/MS analysis. The metabolomics data were normalized by $\log_2$ conversion, optimized by z-score, and then analyzed for differential metabolites using SIMCA 14.1. SIMCA was a principal component analysis (PCA)-based supervised classification technique and was used extensively in the field of metabolomics.¹⁹ The samples of the quality control (QC) were added into the cohort to detect the stability of the instrument. The unsupervised PCA and orthogonal partial least-squares discriminant analysis (OPLS-DA) were used for analyzing the intergroup differences. The efficacy of OPLS-DA models was further verified by permutation tests 200 times. A mixed screening standard of fold change (FC) values, *P*-values, and variable important in projection (VIP) values ($\text{FC} \geq 1.5$ or ≤ 0.67 , $P < 0.05$, $\text{VIP} > 1$) was used for screening the differential metabolites. The quantities of differential metabolites were illustrated by volcano and Venn diagrams. Volcano and Venn diagrams were performed by GraphPad 8.0. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis is a bioinformatics analysis method for identifying biological pathways that are enriched in the KEGG database. According to the KEGG compound database (<http://www.kegg.jp/kegg/compound/>), the identified metabolites were labeled and the labeled metabolites were plotted to the KEGG pathway database (<http://www.kegg.jp/kegg/pathway.html>).²⁰ In KEGG pathway enrichment analysis, the top 20 significantly enriched pathways were calculated based on the *P*-value of enrichment significance of differential metabolites by MSEA (metabolite set enrichment analysis).

For fungal microbiome data analysis, taxonomic identification of features was conducted by the unite_reference_data set (04.02.2020) and the Vsearch consensus taxonomy classifier implemented in Qiime2. Most statistical analyses and data processing were performed using R software. The α -diversity was analyzed based on the number of features. Community composition differences (β -diversity) among samples were calculated based on Bray–Curtis dissimilarities and principal coordinate analysis (PCoA).

In Vitro Validation Experiments of Metabolites Inhibiting Pathogenic Fungi. Swainsonine solution (prepared with sterile water) and apigenin-7-O-glucoside-4-O-rutinoside solution (prepared with methanol) were prepared in a series of dose gradients based on the previous research:^{9,21} low-dosage group (0.005 mg L^{-1}), middle-dosage group (0.025 mg L^{-1}), and high-dosage group (0.125 mg L^{-1}). Solutions of different doses were added to the prepared PDA agar medium and mixed well. For the control group, equivalent sterile water or methanol was added to the PDA agar medium and mixed well. A 5 mm diameter sterile punch was used to intercept a 5 mm mycelial disk from the cultured test strain (*Leptosphaeria sclerotoides* and *Colletotrichum americanae-borealis*) evenly into the above culture medium, which was cutoff from the periphery of the colony in the 7-day cultivated colonies. After 4 days of incubation at 25 $^{\circ}\text{C}$ in the dark, the colony diameters of these mycelial dishes were determined. Each group was repeated three times.

Mechanism Analysis of Swainsonine and Apigenin-7-O-glucoside-4-O-rutinoside Inhibiting Pathogenic Fungi. According to the results of in vitro validation experiments, a high dosage

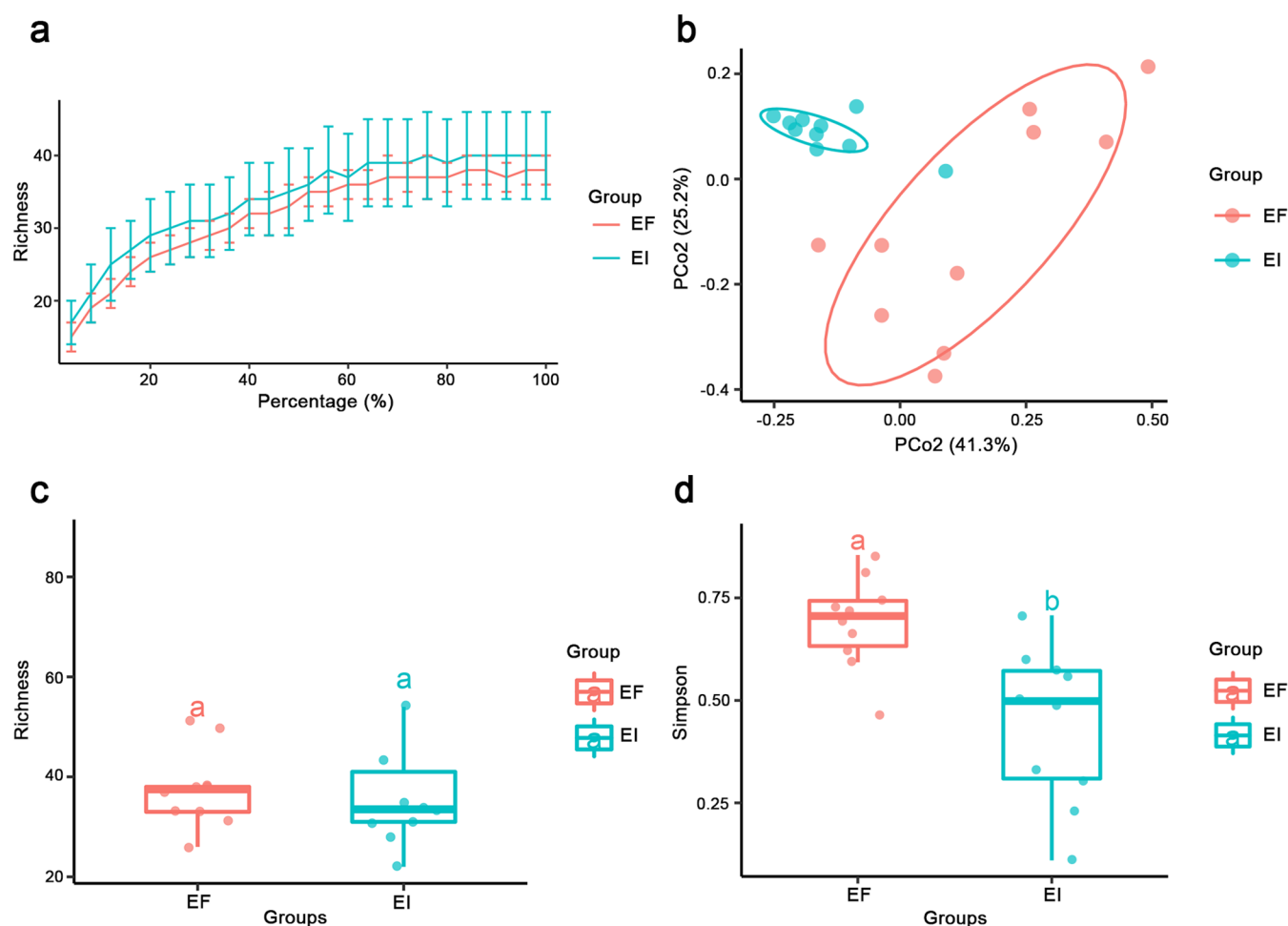


Figure 1. Microbial diversity analysis of the *O. ochrocephala* microbiome between the endophyte-free (EF) group and the endophyte-infected (EI) group. (a) Rarefaction curve of richness. (b) PCoA plot based on the Bray–Curtis matrix of β -diversity. (c) Boxplot of richness indices of α -diversity. (d) Boxplot of Simpson indices of α -diversity. Data are expressed as mean $\pm$ SD; $n = 10$ per group; bar chart (c, d) with different letters on top indicates statistically significant results based on one-way ANOVA analysis ($P < 0.05$).

(0.125 mg L⁻¹) of both swainsonine and apigenin-7-O-glucoside-4-O-rutinoside significantly inhibited the growth of *Leptosphaeria sclerotoides* and *Colletotrichum americanae-borealis*. Thus, the mechanism analysis of these two metabolites inhibiting pathogenic fungi was investigated based on the high dosage (0.125 mg L⁻¹). 200 μ L of swainsonine and apigenin-7-O-glucoside-4-O-rutinoside was separately added to the surface of the cultured test strain (*Leptosphaeria sclerotoides* and *Colletotrichum americanae-borealis*). For the control group, 200 μ L of sterile water was added to the surfaces of the cultured test strain. Each group was repeated three times. The contents of total antioxidant capacity (T-AOC), superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA) were determined by the corresponding kits (Biogoethe Biotechnology Co., Ltd., Wuhan, China).

Data Analysis. We carried out graphic illustration and statistical analysis by utilizing GraphPad software (8.0 version) and R Programming Language (R 4.1.0). A mixed screening standard of FC values, P -values, and VIP values ($FC \geq 1.5$ or ≤ 0.67 , $P < 0.05$, $VIP > 1$) was used for screening the differential metabolites. One-way ANOVA analysis was used to test the significant differences in pairwise comparison of α -diversity analysis and in vitro validation experiments. The boxplots of significant different genera and phylum between EI and EF were analyzed by Wilcox ($P < 0.05$, FDR < 0.1).

RESULTS

Microscopic Observation and Molecular Identification of Endophytic Fungi. For molecular identification,

PCR with the specific primer pair OmtssuF/OmtssuR (Table S1) preliminarily determined 42 EI samples in a total of 60 *O. ochrocephala* samples (Table S2), and the fungal carrier rate was 70% by calculating the ratio of plants containing endophytic fungi to all plants. For microscopic observation, mycelia were visible as blue filaments in stained stem pith of 44 of 60 *O. ochrocephala* plants (Figure S2). Microscopic observation preliminarily determined 44 EI samples in a total of 60 samples (Table S2), and the fungal carrier rate was 73.3% by calculating the ratio of plants containing endophytic fungi to all plants. Plants both positive in microscopy and molecular identification were selected as EI and the contrary for EF. Thus, 10 EI plants (G-2, G-16, G-17, G-22, G-29, G-35, G-41, G-60, G-72, and G-78) and 10 EF plants (G-12, G-39, G-44, G-51, G-52, G-53, G-54, G-61, G-74, and G-75) were selected for subsequent experiments.

High-Throughput Sequencing of ITS of *O. ochrocephala*-Associated Fungi. The composition of the *O. ochrocephala* endophytic fungal community was analyzed by high-throughput sequencing of ITS. The rarefaction curve reached a stabilizing value, indicating that the sequencing depth of our samples was sufficient (Figure 1a). For β -diversity (Figure 1b), the PCoA plot based on the Bray–Curtis matrix indicated a significant difference between the EF and EI groups ($P = 0.00009$; ANOSIM, permutations 999). The α -diversity

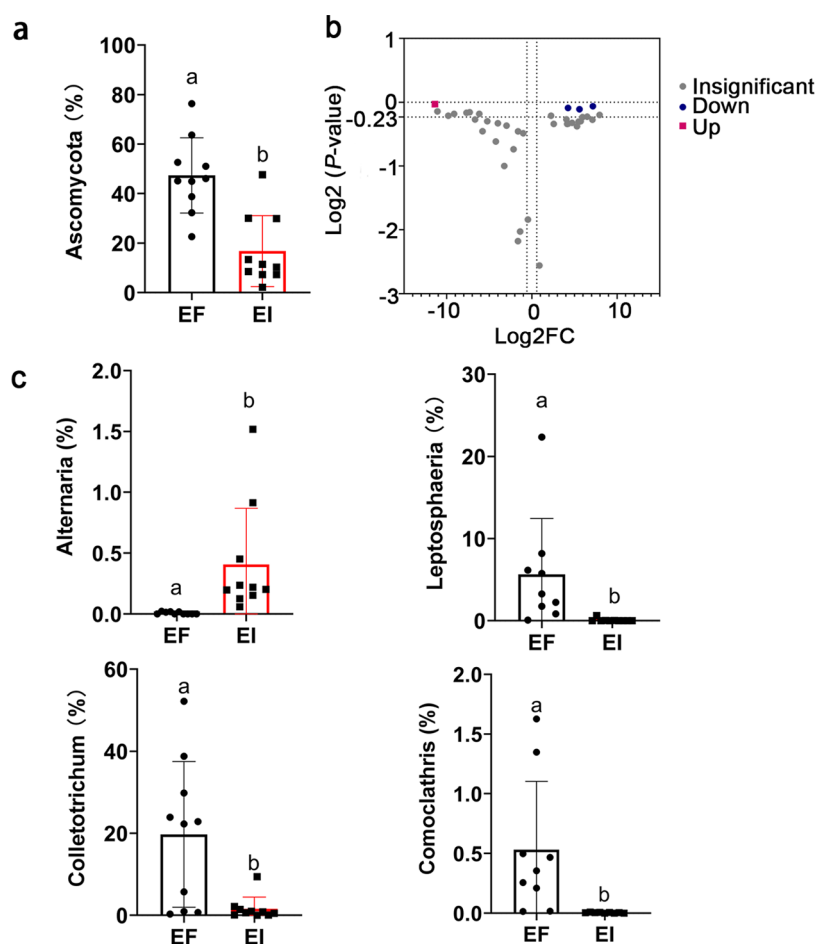


Figure 2. Abundance of phylum Ascomycota and different genera in *O. ochrocephala* EF and EI groups. (a) Boxplot of phylum Ascomycota. (b) Volcano plot of different genera. (c) Boxplots of genus *Alternaria*, *Leptosphaeria*, *Colletotrichum*, and *Comoclathris*. Data are expressed as mean $\pm$ SD; $n = 10$ per group; bar chart (a, c) with different letters on top indicates statistically significant results based on the Wilcoxon analysis ($P < 0.05$, FDR < 0.1).

results indicated that the richness index had no significant difference between the two groups (Figure 1c); however, the Simpson index had a significant difference between the two groups (Figure 1d). At the level of phyla, the dominant phylum of the EF group was Ascomycota, which was significantly lower in the EI group ($P = 0.05$) (Figure 2a). Other phyla included Basidiomycota, Mucoromycota, Mortierellomycota, and Chytridiomycota. Four genera with significant differences between different groups were screened by the volcano plot; three of these genera were downregulated in the EI group, and one was upregulated in the EI group (Figure 2b). Notably, the relative abundance of *A. oxytropis* ($P = 0.01$) was significantly greater in the EI group (Figure 2c), whereas the relative abundance of *Leptosphaeria* ($P = 0.04$), *Colletotrichum* ($P = 0.005$), and *Comoclathris* ($P = 0.04$) was markedly less in the EI group (Figure 2c).

Pseudotargeted Metabolomics Analysis of *O. ochrocephala* Metabolites. We provided detailed information on all metabolites (Table S3). The spectral and chromatographic data for the MS/MS information on 46 random identified metabolites and the match of databases were exhibited in Figure S3. The PCA plot demonstrated differences in metabolites between the two groups, and the quality control (QC) samples were well concentrated together, indicating that the representativeness was good. (Figure 3a). The OPLS-DA score plots exhibited that there was marked separation between

the EF and the EI groups ($R^2X = 0.997$, $Q^2 = 0.772$) (Figure 3b), indicating that the model was reliable and had good predictive ability. The effectiveness of these models was further confirmed by permutation tests (Figure 3c). Volcano plots were utilized to screen the differential metabolites between the two groups (Figure 4a). We found 70 differential metabolites, of which 29 were higher and 41 were lower in the EI group, and all of the relevant information on 70 differential metabolites between the EF and EI groups is provided in Table S4. The Venn plot showed that 25 common differential metabolites were obtained by independent pairwise comparison, 25 unique metabolites were obtained in the EF group, and 20 unique metabolites were obtained in the EI group (Figure 4b). The top 20 metabolic pathways were found by the KEGG pathway enrichment analysis, of which the top 10 were thiamine metabolism, arachidonic acid metabolism, isoflavonoid biosynthesis, biosynthesis of cofactors, sulfur relay system, oxidative phosphorylation, isoquinoline alkaloid biosynthesis, indole alkaloid biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, and riboflavin metabolism (Figure 4c).

Metabolic biomarkers of *O. ochrocephala* between the two groups were screened by the machine learning method's random forest model. According to the relative importance of the metabolites, five important metabolites with the highest variable importance values were selected as potential metabolic biomarkers (Figure 5a). The receiver operating characteristic

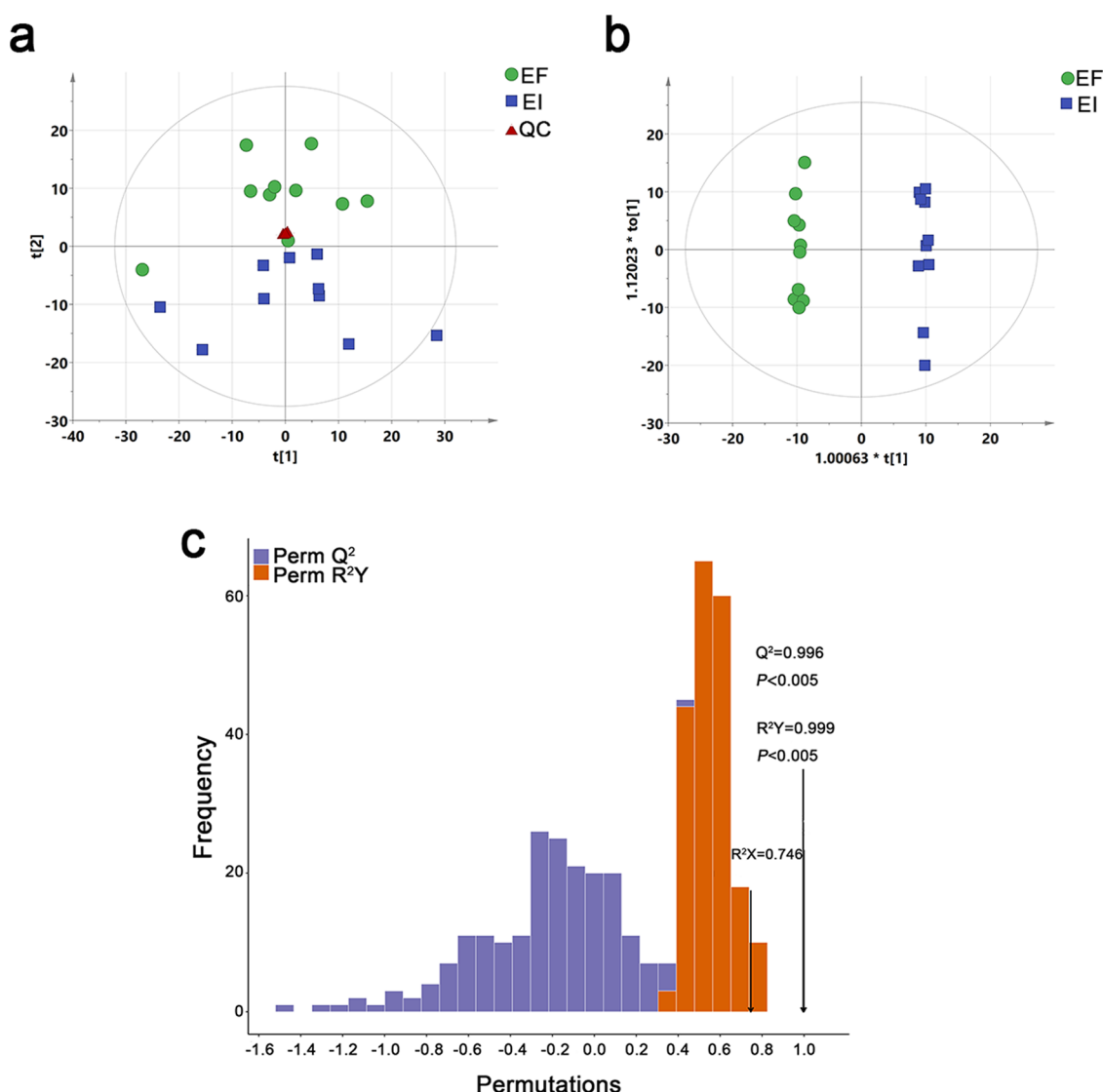


Figure 3. Metabolomic analysis of the *O. ochrocephala* EF and EI groups: (a) PCA plot. (b) OPLS-DA score plot. (c) Permutation plot obtained from the OPLS-DA score plot. R^2Y and Q^2 respectively represent the interpretation rate of the model to the matrix and the prediction ability of the model. R^2Y represented the cumulative explanatory rate of the model in the Y -axis direction, and Q^2 represented the cumulative prediction rate of the model when modeling the multivariate statistical analysis. In theory, when the values of R^2 and Q^2 were closer to 1, indicating a better model, it was acceptable when R^2 and Q^2 were higher than 0.4.

(ROC) curves for these five biomarkers were drawn, and the area enclosed by the ROC curve and the abscissa was a value called area under the curve (AUC), whose value greater than 0.6 indicated that the model was valid. The AUC values of five potential biomarkers for distinguishing the EF and EI groups are shown in Figure 5b, including apigenin-7-O-glucoside-4-O-rutinoside (AUC = 0.950), 2-hydroxy-4-methylpentanoic acid (AUC = 0.950), medicagenic acid-3-O-glucuronide-28-O-xylosyl (1,4)-rhamnosyl (1,2)-arabinoside (Immn005003; AUC = 1.000), isorhamnetin (AUC = 1.000), and swainsonine (AUC = 1.000). As shown in Figure 5c, the relative abundances of apigenin-7-O-glucoside-4-O-rutinoside and swainsonine were markedly higher in the EI group, and the relative abundances of 2-hydroxy-4-methylpentanoic acid, Immn005003, and isorhamnetin were significantly lower in the EI group.

Correlation Analysis of Metabolites and Fungal Community. A histogram of the quantitative analysis by targeted metabolomics reflected that the quantitative concen-

tration of swainsonine was notably higher in the EI group compared with the EF group (Figure 6a). The abundance of endophytic fungi *A. oxytropis* in each sample was obtained by high-throughput sequencing of ITS. Spearman's correlation indicated that the abundance of *A. oxytropis* producing swainsonine was significantly positively correlated with the concentration of swainsonine ($R^2 = 0.9346$, $P < 0.0001$) (Figure 6b).

In Vitro Validation Experiments of Metabolites Inhibiting Pathogenic Fungi. Apigenin-7-O-glucoside-4-O-rutinoside and swainsonine as potential biomarkers were significantly elevated in the EI group, and pathogenic fungi including *Leptosphaeria* and *Colletotrichum* were markedly decreased, suggesting these two biomarkers were closely related to pathogenic fungi. To verify the inhibitory effect of apigenin-7-O-glucoside-4-O-rutinoside and swainsonine on pathogenic fungi, we added different doses of these two metabolites into the medium of culturing *Leptosphaeria sclerotoides* or *Colletotrichum americanae-borealis*. We found that

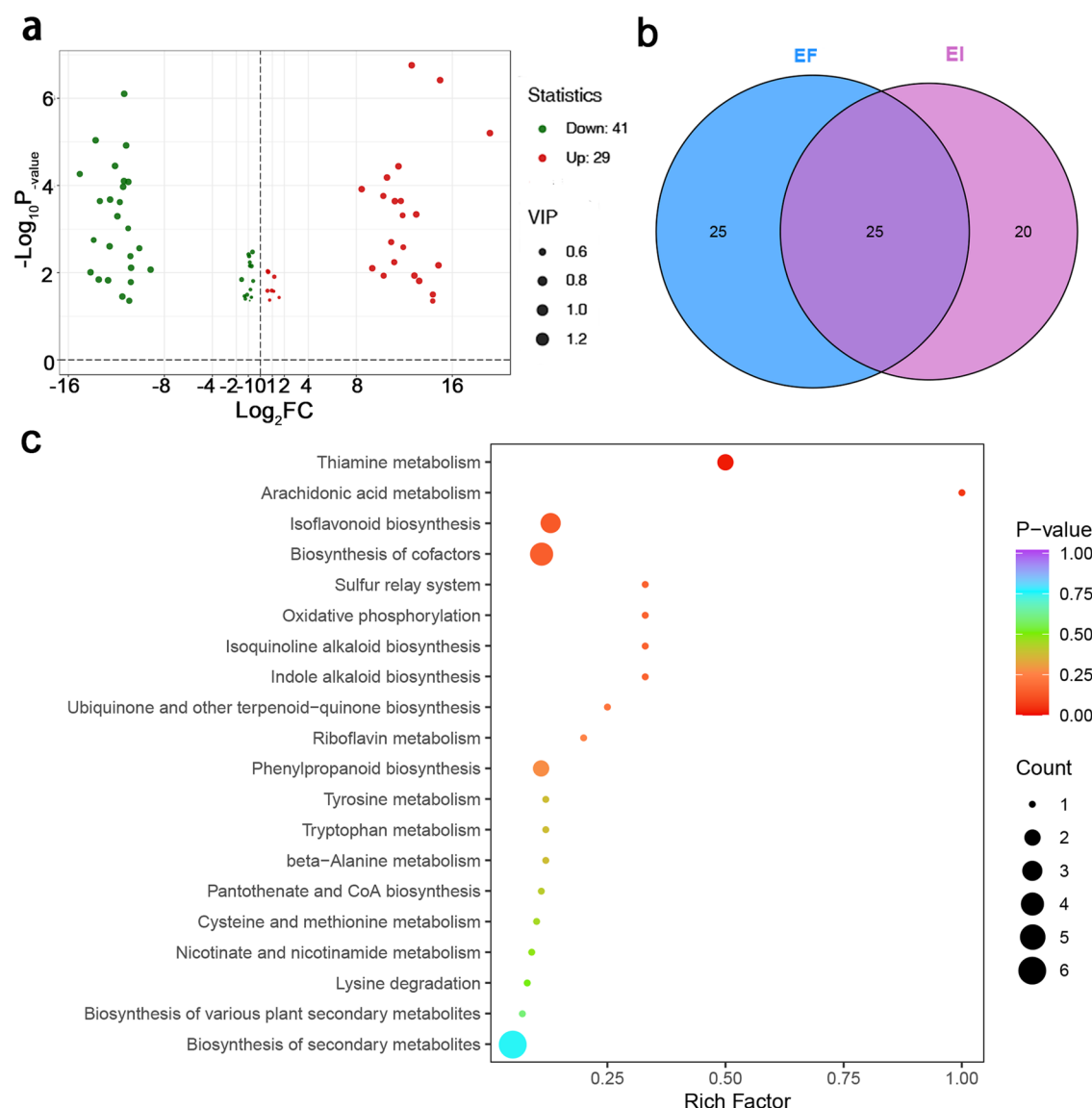


Figure 4. Volcano plot, Venn chart, and KEGG pathway enrichment analysis of differential metabolites of the *O. ochrocephala* EF and EI groups. (a) Volcano plot of EI vs EF. (b) Venn chart showed the common differential metabolites and unique differential metabolites between the EI and the EF groups. (c) Metabolic pathways of differential metabolites between the EI and the EF groups by KEGG pathway enrichment analysis.

high doses of swainsonine and apigenin-7-O-glucoside-4-O-rutinoside treatments significantly reduced the colony diameter of *Leptosphaeria sclerotoides* (Figure 7a,b). Low, middle, and high doses of apigenin-7-O-glucoside-4-O-rutinoside significantly inhibited the growth of *Colletotrichum americanae-borealis* (Figure 7c). Middle and high doses of swainsonine significantly inhibited the growth of *Colletotrichum americanae-borealis* (Figure 7d).

Mechanism Analysis of Swainsonine and Apigenin-7-O-glucoside-4-O-rutinoside Inhibiting Pathogenic Fungi. We measured the oxidative stress response of *Leptosphaeria sclerotoides* or *Colletotrichum americanae-borealis* under swainsonine and apigenin-7-O-glucoside-4-O-rutinoside treatments. For *Leptosphaeria sclerotoides*, the activity of T-AOC was significantly increased in the swainsonine group compared with the control and apigenin-7-O-glucoside-4-O-rutinoside groups. The activity of SOD was significantly upregulated after the treatment with swainsonine and apigenin-7-O-glucoside-4-O-rutinoside compared to the control group.

The activity of CAT was markedly elevated in the swainsonine group compared to the control group. The MDA activity was remarkably higher after treatment with swainsonine and apigenin-7-O-glucoside-4-O-rutinoside (Figure 8a).

For *Colletotrichum americanae-borealis*, compared with the control group, two metabolite treatments significantly increased T-AOC activity ($P < 0.001$). The SOD activity was dramatically elevated after the treatment with swainsonine compared to the control and apigenin-7-O-glucoside-4-O-rutinoside-treated groups. CAT activity did not change significantly after two metabolite treatments, but there was an upward trend. It should be noted that the activity of MDA was significantly higher under swainsonine and apigenin-7-O-glucoside-4-O-rutinoside treatments than under the control group (Figure 8b).

DISCUSSION

Endophytic fungi are vital components of plant microbiota. They can affect plant growth and improve plant resistance by

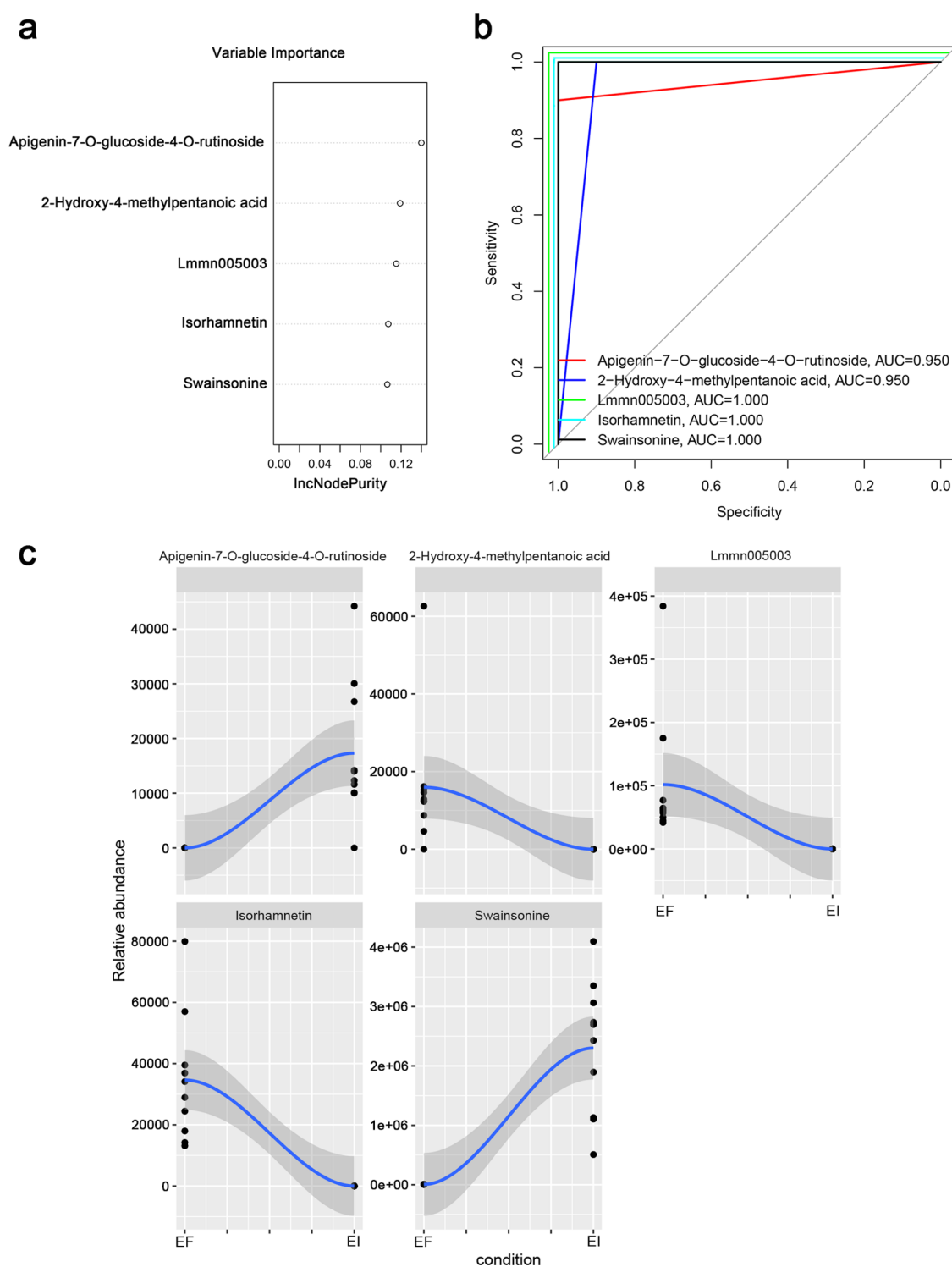


Figure 5. Machine learning and receiver operating characteristic curve analysis. (a) Five metabolic biomarkers of the *O. ochrocephala* EF and EI groups by the random forest model. (b) ROC curves of five biomarkers. (c) Fitting curve plot of five biomarkers between the EI and the EF groups. Lmmn005003 represents medicagenic acid-3-O-glucuronide-28-O-xylosyl (1,4)-rhamnosyl (1,2)-arabinoside.

producing secondary metabolites. Dhayanithy et al.²² found that endophytic fungi in *Catharanthus roseus* could produce some secondary metabolites with biological activities such as eliminating free radicals. *Beauveria bassiana*, an endophytic fungus in northern corn (*Zea mays* L.), inhibited the relative abundance of phytopathogenic fungi *Exserohilum turcicum* by increasing the plant beneficial bacteria.²³ *A. oxytropis* dominated the endophytic fungi in many locoweeds. With

the increase of the abundance of *A. oxytropis*, the microbial diversity indexes (Shannon and Simpson) of *Nicotiana tabacum* cultivar K326 leaf were significantly changed, and five pathogenic bacteria were inhibited by *A. oxytropis*.²⁴ However, the effect of *A. oxytropis* on the microbiome and metabolome of *O. ochrocephala* remained undetermined. In the present study, based on the joint analysis of high-throughput sequencing of ITS and LC-ESI-MS/MS results, the fungal

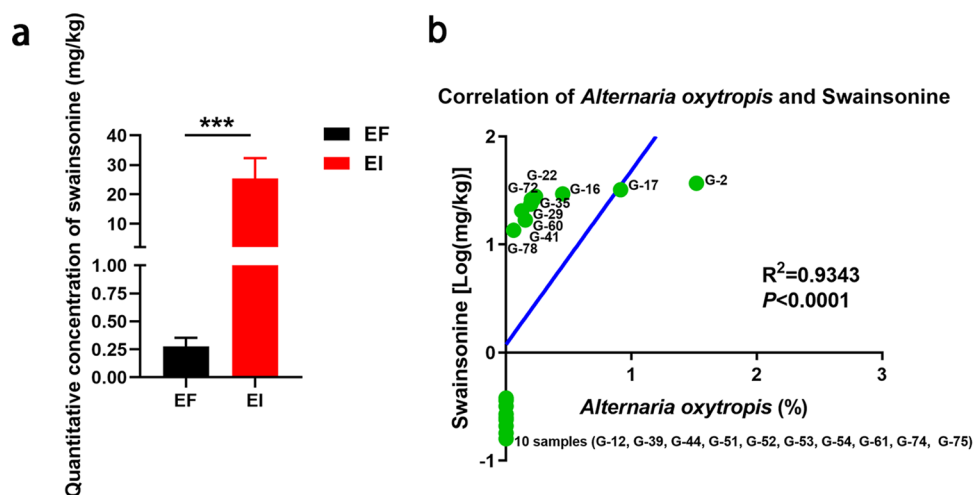


Figure 6. Correlation analysis of *A. oxytropis* and swainsonine. (a) Quantitative concentration of swainsonine between the *O. ochrocephala* EF and EI groups. (b) Correlation scatter plot between *A. oxytropis* and swainsonine. Data are presented as mean $\pm$ SD; $n = 10$ per group; *** on the top of bar chart (a) indicates statistically significant results based on one-way ANOVA analysis ($***P < 0.001$).

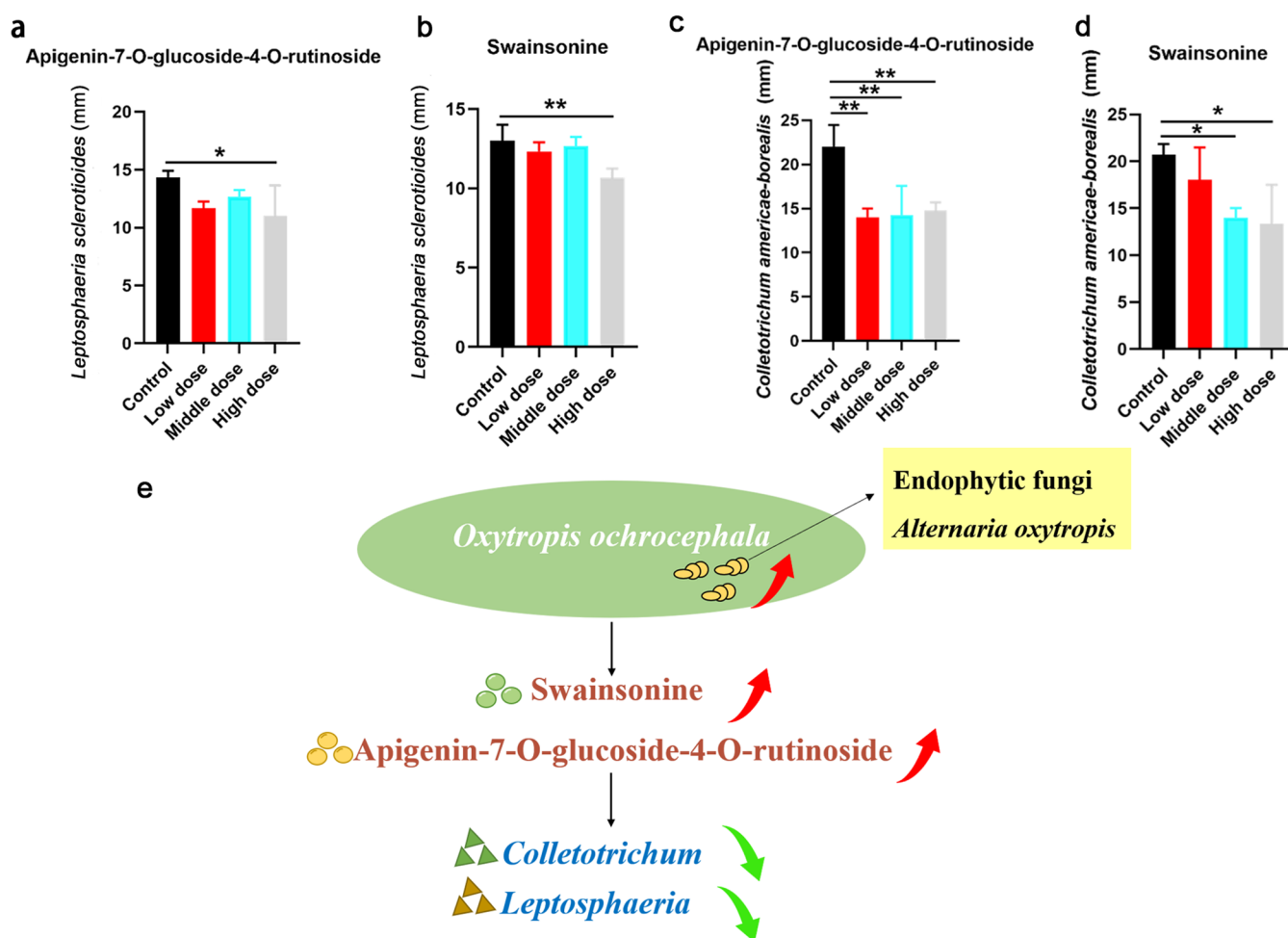


Figure 7. In vitro validation experiments of different doses of metabolites inhibiting pathogenic fungi. (a, b) Colony diameter of *Leptosphaeria sclerotioidea* under different doses of apigenin-7-O-glucoside-4-O-rutinoside and swainsonine. (c, d) Colony diameter of *Colletotrichum americanae-borealis* under different doses of apigenin-7-O-glucoside-4-O-rutinoside and swainsonine. (e) Interrelationship diagram between endophytic *A. oxytropis* and pathogenic fungi in *O. ochrocephala*. Data are presented as mean $\pm$ SD; $n = 10$ per group; differences of data in different groups were assessed by one-way ANOVA analysis ($*P < 0.05$, $**P < 0.01$).

community and metabolites of *O. ochrocephala* were compared between the EF and EI groups.

Our α -diversity results showed that *A. oxytropis* endophyte presence in *O. ochrocephala* significantly decreased the

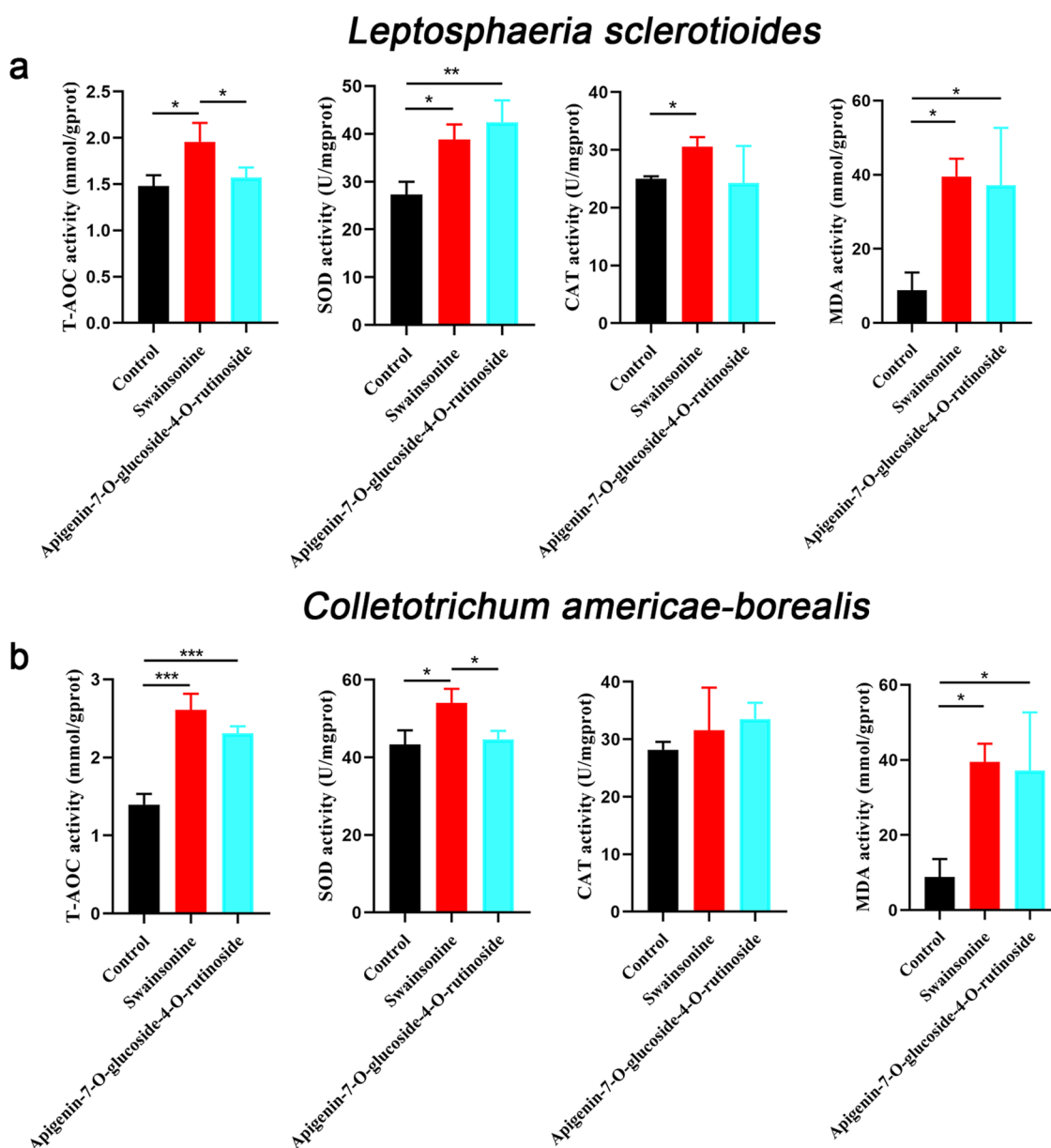


Figure 8. Mechanisms underlying the inhibition of pathogenic fungi by swainsonine and apigenin-7-O-glucoside-4-O-rutinoside. (a) Activities of T-AOC, SOD, CAT, and MDA in *Leptosphaeria sclerotioides* under the treatments of swainsonine or apigenin-7-O-glucoside-4-O-rutinoside. (b) Activities of T-AOC, SOD, CAT, and MDA in *Colletotrichum americae-borealis* under the treatments of swainsonine or apigenin-7-O-glucoside-4-O-rutinoside. Data are presented as mean $\pm$ SD; $n = 10$ per group; differences of data in different groups were assessed by one-way ANOVA analysis (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Simpson index of the fungi community (Figure 1d), but had no significant effect on the richness index (Figure 1c). The previous study indicated that the abundance *A. oxytropis* was markedly negatively associated with fungal richness,²⁵ which was consistent with our results. The results of β -diversity indicated that the presence of *A. oxytropis* had a remarkable impact on the fungal community structure of *O. ochrocephala*. Our results further supported the hypothesis previously mentioned by Liu et al.¹⁶ that endophytic fungi could regulate the diversity and community structure of host plant bacterial and fungal communities. Ascomycota was closely related to root rot in *Panax notoginseng*.²⁶ Less abundant Ascomycota in the EI group exhibited that the presence of *A. oxytropis* decreased the risk of *O. ochrocephala* being infected with fungal diseases of the Ascomycota phylum in this study. *Leptosphae-*

ria,²⁷ *Colletotrichum*,²⁸ and *Comoclathris*¹² are well-known plant pathogens. *Leptosphaeria* can induce diseases of rape²⁹ and other plants, causing serious economic losses, and many toxic secondary metabolites have been isolated from *Leptosphaeria*-infected plants. Because of its wide range of parasitism, *Colletotrichum* is widely distributed and can infect crops and medicinal plants.¹⁰ It has been speculated that *Comoclathris* may be an important pathogenic fungus causing *Bromus tectorum* stand failure.¹² Our results stated that these three pathogenic fungi, *Leptosphaeria*, *Colletotrichum*, and *Comoclathris*, were significantly upregulated in the EF group, indicating that *O. ochrocephala* without endophytic fungi was susceptible to disease. The presence of endophytic *A. oxytropis* would significantly reduce the risk of plant infection with these three pathogenic fungi, thus improving plant disease resistance

and indicating that the antifungal ability of *O. ochrocephala* was closely related to the level of *A. oxytropis*. Consistently, improved plant disease resistance by *A. oxytropis* was an effective method for controlling fungal diseases of *O. ochrocephala*.

In our study, the significantly enriched differential metabolic pathways between the EF and EI groups were identified by the KEGG pathway enrichment analysis, according to differential metabolites. It was found that the significantly enriched metabolic pathways mainly included thiamine metabolism, arachidonic acid metabolism, biosynthesis of cofactors, sulfur relay system, oxidative phosphorylation, and indole alkaloid. Thiamine metabolism plays a fundamental role in the abiotic and biotic stresses of plants.³⁰ Studies have found that arachidonic acid metabolism mediates enhancement of plant tolerance to pathogens by activating general stress-response genes.³¹ Vitamin B6³² and iron–sulfur cluster³³ are essential cofactors for metabolic enzymes, which are involved in defense against stress responses, respectively. For aerobic cells, oxidative phosphorylation is an important process, which produces about 90% of cellular ATP.³⁴ Indole alkaloids, synthesized from tryptophan or other indole donors, have attracted much attention due to their medicinal value. Additionally, certain indole alkaloids have defensive function, including against pests and pathogens in plants.³⁵ It is worth mentioning that ubiquinone and other terpenoid–quinone biosynthesis is proportional to the ability to avoid ROS damage.³⁶ Riboflavin is an essential nutrient for plants and animals. Thus, it is suggested that the presence of endophytic *A. oxytropis* may alter these important metabolic pathways in *O. ochrocephala*, which are related to stress response and disease resistance of plants.

Alternaria inhibited pathogenic fungi and had a reversal relationship with pathogens. We hypothesized that *A. oxytropis* may improve antifungal properties in the host plant by increasing the inhibitory metabolite levels of pathogenic fungi. In the current study, five important metabolites were recognized as the potential metabolic biomarkers to distinguish the EF group and EI group by the random forest model. Among them, the relative abundance of apigenin-7-O-glucoside-4-O-rutinoside and swainsonine were significant higher, and 2-hydroxy-4-methylpentanoic acid, lmmn005003, and isorhamnetin were significant lower in the EI group (Figure 6c). Swainsonine as a secondary metabolite can inhibit the invasion of foreign pathogens including pathogenic fungi.³⁷ Apigenin, a natural plant-derived flavonoid, can provide important antifungal activity and can even promote plant growth and improve plant resistance by activating the defense system.³⁸ 2-Hydroxy-4-methylpentanoic acid is the phytotoxin associated with blackleg disease, which is destructive to several economical crops.³⁹ Here, the increase in the relative abundance of swainsonine and apigenin-7-O-glucoside-4-O-rutinoside and the decrease in the relative abundance of 2-hydroxy-4-methylpentanoic acid suggested that the endophytic *A. oxytropis* can improve the antifungal capacity of *O. ochrocephala*. However, isorhamnetin with antimicrobial activity⁴⁰ was significantly lower in the EI group. In addition, the specific role of lmmn005003 in plants was still unclear. Changes in the metabolites can reflect the results of internal plant regulation. In the previous study, the *Epichloë gansuensis* elevated vanillin content and decreased pyruvate content in leaves to improve the tolerance of *Achnatherum inebrians* under low P stress.⁴¹ Interestingly, our results provided direct

evidence that swainsonine and apigenin-7-O-glucoside-4-O-rutinoside can significantly inhibit the growth of pathogenic fungi such as *Leptosphaeria sclerotoides* and *Colletotrichum americanum-borealis*. Our results suggested that *O. ochrocephala* symbiotic with the endophytic *A. oxytropis* could increase the resistance to fungal diseases by markedly enhancing the levels of metabolites inhibiting pathogenic fungi.

Oxidative stress was produced when the metabolism of reactive oxygen species was not balanced. When fungi were suffering from stress, it can produce reactive oxygen species (ROS) that bind to proteins, lipids, and DNA, leading to an increase in plant membrane permeability and a decrease in enzyme activity, disrupting normal metabolic homeostasis.⁴² Modulation of antioxidant levels was able to counteract stress mainly by increasing the synthesis of T-AOC, SOD, CAT, and MDA.⁴³ T-AOC was a comprehensive index for evaluating the ability of antioxidants, which can reflect the antioxidant activity of the organism and in general reflect the strength of the antioxidant capacity of the defense system. Therefore, T-AOC was the sum of all antioxidant properties, which can reflect the functional state of the total defense capacity.⁴⁴ SOD mainly catalyzed the transformation of superoxide radicals into hydrogen peroxide (H_2O_2) and was the first antioxidant defensive fraction for removing oxygen radicals. CAT can effectively regulate H_2O_2 levels and played an essential role in the prevention of oxidative stress.⁴⁵ MDA was one of the final degradation products formed by reactive oxygen species attacking membrane lipids, and its level can reflect the magnitude of cellular damage.⁴⁶ In previous studies, the levels of these four indicators of oxidative stress increased significantly when the fungi were stressed.^{43,45,47} In our study, the elevated T-AOC, SOD, CAT, and MDA activities indicated that the antioxidant system of the pathogenic fungi was disrupted, thereby inhibiting the growth of pathogenic fungi by the treatment of swainsonine and apigenin-7-O-glucoside-4-O-rutinoside.

It is worth noting that with the increase of the abundance of endophytic *A. oxytropis*, the content of its metabolite swainsonine was also markedly increased (Figure 6), which would further affect the health level of livestock due to the toxicity of the alkaloid. Previous studies have shown that the level of swainsonine was related to the abundance of endophytic fungi within the host population.⁴⁸ Similarly, the hypothesis that the abundance of endophytic *Alternaria* was proportional to the content of swainsonine was also supported by the study of Cook et al.⁹

Integrated multiomics technologies can reveal key cell signaling compounds for plant stress resistance and promote sustainable agricultural production.⁴⁹ In this study, swainsonine and apigenin-7-O-glucoside-4-O-rutinoside, metabolic biomarkers screened from pseudotargeted metabolomics, significantly inhibited the growth of pathogenic fungi *Leptosphaeria sclerotoides* and *Colletotrichum americanum-borealis* by increasing the level of oxidative stress in fungi. Collectively, the endophytic *A. oxytropis* enhanced the ability to inhibit pathogenic fungi in *O. ochrocephala* by markedly elevating the concentrations of swainsonine and apigenin-7-O-glucoside-4-O-rutinoside (Figure 7e). This study may provide a basis and new insight for the utilization of agricultural resources of *O. ochrocephala*, suggesting that utilization of ecologically adapted locoweed may alleviate the shortage of agricultural forages.

■ ASSOCIATED CONTENT

Data Availability Statement

Raw sequence reads have been deposited in the National Center for Biotechnology Information Sequence Read Archive (accession number: PRJNA887584).

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.3c09049>.

High-throughput sequencing of ribosomal internal transcribed spacer (ITS) (Methods S1) and pseudotargeted metabolomics analysis (Methods S2); specific PCR primers used for *Mtssu* gene and ITS (Table S1); microscopic observation and molecular identification of endophytic fungi from 60 plants of *Oxytropis ochrocephala* (Table S2); differential metabolites between the EF group and the EI group (Table S4); flowchart of experimental setup (Figure S1); microscopic observation of endophytic fungi treated with aniline blue of *O. ochrocephala* (Figure S2); and spectral and chromatographic data for the MS/MS information on identified metabolites and the match of databases (Figure S3) (PDF)

Detailed information on all metabolites *O. ochrocephala* (Table S3) (XLSX)

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K.F.: Writing original draft manuscript, investigation, data curation, validation, visualization. C.L.S.: Writing-review and editing. D.C.: Methodology, writing-review and editing. X.C.: Investigation, resources. N.L.: Data curation, writing-review and editing. C.H.: Investigation. D.W.: Investigation. L.X.: Resources. Y.L.: Writing-review and editing, funding acquisition, supervision. Z.S.: Writing-review and editing, experimental design, funding acquisition, investigation, validation, visualization.

Notes

The authors declare no competing financial interest.

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