Hindawi
Journal of Chemistry
Volume 2022, Article ID 7613285, 7 pages
https://doi.org/10.1155/2022/7613285

Research Article

Metabolomics Study on the Resistance of Walnut Peel to *Colletotrichum gloeosporioides* under Prochloraz Treatment

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Received 29 April 2022; Accepted 25 May 2022; Published 28 June 2022

Academic Editor: Pei Li

Anthracnose, caused by *Colletotrichum gloeosporioides*, is highly harmful for walnut production in the world. To better control this disease, the inhibitory effects of 3 fungicides against *Colletotrichum gloeosporioides* were determined, and the results showed that prochloraz had better inhibitory activity. Through comparative metabolomics analysis, 311 metabolites might be associated with the walnut peel response to *Colletotrichum gloeosporioides* under prochloraz treatment. Furthermore, we supposed that the phenylpropanoid pathway might be induced by prochloraz to resist *Colletotrichum gloeosporioides* infection. In conclusion, the upregulated metabolites in the phenylpropanoid pathway might be related to synthesize lignin to further form a cell wall against *Colletotrichum gloeosporioides* infection.

1. Introduction

Colletotrichum gloeosporioides (*C. gloeosporioides*) is a plant pathogen highly harmful for many genus plants [1–3]. It can cause anthracnose disease which is a crucial constraint to walnut production in many areas in the world and results in significant yield and economic losses [4, 5]. Some reports demonstrated that some fungicides can inhibit anthracnose disease caused by *C. gloeosporioides* [6, 7], and prochloraz is a good fungicide to control diseases, which can inhibit the ergosterol synthesis of pathogen. However, there was a difference pathogenicity from different isolates. It should consider that the pathogenicity of the genus *Colletotrichum* from different sources [8] and these strains isolates from different plant or place had different fungicides sensitivities too [1, 2]. Therefore, any differences in fungicide sensitivities of isolates causing walnut anthracnose remain unknown [9]. Walnut anthracnose caused walnut production economy loss in Guizhou province every year, and the main pathogen is *C. gloeosporioides*, in which fungicide fit for this strain on walnut is unknown. It is known that prochloraz can inhibit the ergosterol synthesis of pathogen, but how it effects metabolic changes of walnut peels is unknown. This study was conducted to compare the control effect on *C. gloeosporioides* of walnut and analyze metabolomics of walnut peel against *C. gloeosporioides* under prochloraz treatment.

2. Materials and Methods

2.1. Experimental Materials. The *C. gloeosporioides* was isolated from walnut orchard in Qingzhen City and identified by Guizhou Academy of Forestry. 45% prochloraz EW was purchased from Hunan Xinchangshan Agricultural Development Co., Ltd. (Hunan, China); 50% fluazinam EC was purchased from Mengzhou Nonghuize Biological Technology Co., Ltd. (Henan, China); 80% ethylicin SC was purchased from Shandong Zouping pesticide Co., Ltd. (Shandong, China). The walnut peel was collected from walnut orchard located at Qingzhen City, Guizhou, China (106.276520N, 26.643765E, altitude 1294.77 m).

2.2. Antifungal Activity Test. The effects of three fungicides on the mycelial growth were detected according to the method described by Hua et al. with slight modifications [10]. The fungicides (200, 100, 50, 25, 12.5, and 6.25 mg/L) were prepared using sterile water. The *C. gloeosporioides*

mycelial disks (5 mm in diameter) taken from one-week-old cultures of *C. gloeosporioides* were placed at the center of Petri dishes (90 mm in diameter) containing 20 mL of PDA amended with different concentrations of fungicides. A control experiment was carried out by adding the corresponding volume of sterile water into PDA. Colony diameters were measured after incubation for 7 days at 25°C. Each treatment composed of three replicates. The inhibition rates of fungicides on the colony growth of *C. gloeosporioides* were calculated. The data were presented as the mean ± SD and analyzed to detect significant differences by ANOVA with SPSS version 24.0 software.

2.3. Fungicides Control Efficiency against C. gloeosporioides in the Field. Three fungicides were used to control *C. gloeosporioides* on walnut in Qingzhen City, Guizhou, China (106.276520N, 26.643765E, altitude 1294.77 m). These fungicides were sprayed on 10 walnut trees that were 10 years old; the application time was April 20, May 20, and June 20, 2021, respectively. The drug concentration of fungicides was 1 g/L and water served as blank control (CK). Randomly surveyed one branch from the east, south, north, and west of the trees and counted the area of fruit disease spots on each branch on April 19 and July 20, 2021. At least 20 fruits were surveyed with each tree; each treatment composed of 10 trees. The disease index was calculated according to the disease level. The disease grades were classified as follows. Grade I: no disease spots on the fruit, assigned a value of 0. Grade II: disease spots were less than 10% of the fruit area, assigned a value of 1. Grade III: disease spots were between 10% and 30% of the fruit area, assigned a value of 2. Grade IV: disease spots are between 30% and 50% of the fruit area, assigned a value of 3. Grade V: disease spots are more than 50% of the entire fruit area, assigned a value of 4. Incidence rate = (number of diseased branches/total number of fruits under investigation) × 100; disease index = (∑(disease grades × number of fruits in each grade)/(highest disease grade × total number of investigated fruits)) × 100. Control effect (%) = (1−(disease index after treating−disease index before treating)/control experiment disease index change) × 100. All data were calculated by Excel 2007.

2.4. Metabolomics Analysis. The walnut peels were collected on July 20, 2021. Choose infected fruits with the same symptoms to test. Untargeted metabolomics was used to analysis of the different metabolites of walnut peel between blank control (yunxin1, yunxin2, and yunxin3 named CK) and prochloraz treatment (yunxin4, yunxin5, and yunxin6 named PT). The freeze-dried sample was crushed, and 50 mg of powder was extracted overnight at 4°C with 1 mL of methanol-acetonitrile water (2 : 2 : 1). In addition, quality control (QC) samples were made by mixing the extracts of each sample. The sample extracts were tested by using a Waters Acquity I-Class PLUS/Xevo G2-XS QT of system. The analytical conditions were as follows: column, Waters Acquity UPLC HSS T3 (1.8 μm, 2.1 mm × 100 mm). The following gradient profile was used: 0–2 min, 98% A; 2–11 min, 2%–98% B; 11–13 min, 98% B; and 13–15 min, 2%–98% A. The flow rate was 0.2 mL/min, and the injection volume for each sample was 10 μL. The column temperature was maintained at 40°C, and the sample temperature was 10°C. The injection volume was 3 μL. The effluent was alternatively connected to an Xevo G2-XS QTOF mass spectrometer. Applied a high-resolution tandem mass spectrometer Xevo G2-XS QTOF (Waters, USA), equipped with an ESI Turbo ionspray interface, operating in positive and negative ion modes, and controlled by Analyst MassLynx V4.2. The ESI source operation parameters were as follows: ion source, turbo spray; source temperature, 150°C; ion spray voltage, 2000 V (positive ion mode)/−1500 V (negative ion mode); the sampling cone voltage was set at 30 V; reverse blowing flow rate 50 L/h; and the flow rate of desolvation gas is 800 L/h.

The identification is based on the Progenesis QI software according to the sample type using online databases such as METLIN and self-compiled database (Biomarker, Beijing, China), and the mass deviation of fragment ion identification was within 100 μg/L. Compounds were identified based on second-level mass spectrum and retention time. Quantitative analysis was carried out in the MRM mode. The corresponding relative metabolite contents were expressed as chromatographic peak area integrals. The data were statistically analyzed and plotted using SPSS Statistics version 20.0 (SPSS Inc., Chicago, IL, USA). A principal component analysis (PCA) was performed in R package (version 3.5.0). Orthogonal partial least-squares discriminant analysis (OPLS-DA) was conducted by MetaboAnalystR package (version1.0.1), and the variable importance in projection (VIP) value was generated. Differentially accumulated metabolites (DAMs) were screened by VIP ≥ 1, $P < 0.05$, and |fold change| > 1. The functions of DAMs were further annotated using the KEGG compound database to determine the metabolic pathways that are most highly correlated with the resistance of walnut peel under prochloraz treatment against *C. gloeosporioides*.

3. Results and Discussion

3.1. The In Vitro Antifungal Activity Test. Table 1 provides that 45% prochloraz EW had the best in vitro inhibitory effect on the mycelial growth, with the EC_{50} value of 0.067 μg/mL; it was similarly reported by Ding et al. [7, 11]. The 80% ethylicin SC had the lowest inhibitory effect against *C. gloeosporioides* (EC_{50} = 31.62 μg/mL), which is consistent with Shi et al. report [12].

3.2. Control Efficiency of 3 Fungicides against C. gloeosporioides in the Field. Three fungicides were used to control *C. gloeosporioides* in the field, and the results are given in Table 2. The 45% prochloraz EC had the best control efficiency with 73.28%, 50% fluazinam EC had the control efficiency with 60.34%, and 80% ethylicin SC had the control

Table 1: The in vitro inhibitory effect of 3 fungicides.

Treatment	Concentration (μg/mL)	Colony diameter (cm)	Inhibition rate (%)	EC_{50} (μg/mL)
45% prochloraz EW	200	0.50 ± 0.00	100.00 ± 12.51	0.067
	100	0.65 ± 0.05	97.34 ± 6.57	
	50	0.77 ± 0.07	95.27 ± 5.92	
	25	0.89 ± 0.06	93.08 ± 12.82	
	12.5	0.95 ± 0.05	92.01 ± 4.53	
	6.25	1.18 ± 0.09	87.87 ± 4.18	
50% fluazinam EC	200	0.73 ± 0.07	95.95 ± 6.19	12.76
	100	1.13 ± 0.14	88.76 ± 5.27	
	50	1.70 ± 0.10	78.70 ± 3.09	
	25	2.32 ± 0.11	67.75 ± 2.40	
	12.5	3.38 ± 0.09	48.82 ± 1.10	
	6.25	4.25 ± 0.13	33.43 ± 1.23	
80% ethylicin SC	200	1.20 ± 0.08	87.57 ± 2.57	31.62
	100	2.37 ± 0.12	66.86 ± 1.92	
	50	2.98 ± 0.13	55.92 ± 1.69	
	25	3.78 ± 0.07	41.72 ± 0.83	
	12.5	4.38 ± 0.07	31.07 ± 0.75	
	6.25	4.78 ± 0.09	23.96 ± 0.86	
CK	—	6.13 ± 0.20	—	

Table 2: Control efficiency of 3 fungicides against *C. gloeosporioides* in the field.

Fungicides	Total branches	Disease branches	Disease branch rate (%)	Disease index (%)	Prevention and control effect (%)
45% prochloraz EW	40	30	75	15.50	73.28
50% fluazinam EC	40	36	90	23.00	60.34
80% ethylicin SC	40	40	100	38.00	34.48
CK	40	40	100	58.00	

efficiency with 34.48%. The control efficiency test in the field was consistent with the indoor toxicity test.

The 45% prochloraz EC and 50% fluazinam EC had a better control effect in the field, prochloraz often mixed with other fungicides to obtain better efficiency in the field [13, 14], and fluazinam showed a better inhibition against mycelial growth, but it was unstable and affected by the environment in the field experiment [15]. The ethylicin was the biopesticides; it can inhibit *Botrytis cinerea* effectively but have a little effect on *Colletotrichum* spp. [16].

3.3. Metabolome Analysis Resistance of Walnut under Prochloraz Treatment against C. gloeosporioides. Untargeted metabolomics was performed on samples taken from CK and PT walnut peel after treating on the 30th dpi. A total of 1256 metabolites were obtained in all samples, as presented in the heatmap visualization, which showed distinct hierarchical clustering of samples by different treatment of walnut peel (Figure 1, Table S1). In the PCA plot, CK and PT were divided into two parts by PCA1; it showed different metabolites under two treatments (Figure 2). Based on the quantitative analyses of all detected metabolites (Table S2) and the fold change threshold, a total of 311 DAMs were obtained for the comparison of CK versus PT, of which 68 metabolites were downregulated and 243 metabolites were upregulated in PT. A heatmap of DAM confirmed the significant differences in the metabolome of CK and PT walnut peels following *C. gloeosporioides* infection (Figure 1(b)) [17].

KEGG pathway analysis showed that DAMs were mainly enriched in the following 4 pathways: "biosynthesis of various secondary metabolites," "phenylpropanoid biosynthesis," "folate biosynthesis," and "Biosynthesis of unsaturated fatty acids" (Figure 3). Of these, metabolites involved in the pathways of "phenylpropanoid biosynthesis" showed the highest accumulation.

3.4. DAMs Analysis Dominated Resistance of Walnut under Prochloraz against C. gloeosporioides. All the number of DAMs enriched in the KEGG pathway were 51. Compared DAMs in CK with PT were mainly enriched in the phenylpropanoid pathway. The phenylpropanoid pathway is a well-known metabolite pathway that plays important roles in the plant disease resistance pathway [18]. There were 7 compounds of DAMs in the phenylpropanoid pathway had been detected, and they were all upregulated (Figure 4). The phenylpropanoid KEGG pathway is shown in Figure 5 (Figure S1); sinapic acid, caffeoyl quinic acid, caffeic acid, 5-hydroxyconiferyl alcohol, sinapyl alcohol, ferulic acid, and scopoletin were upregulated in this pathway. These compounds can further synthesize scopoline, sinapoyl malate, syringin, and syringyl lignin.

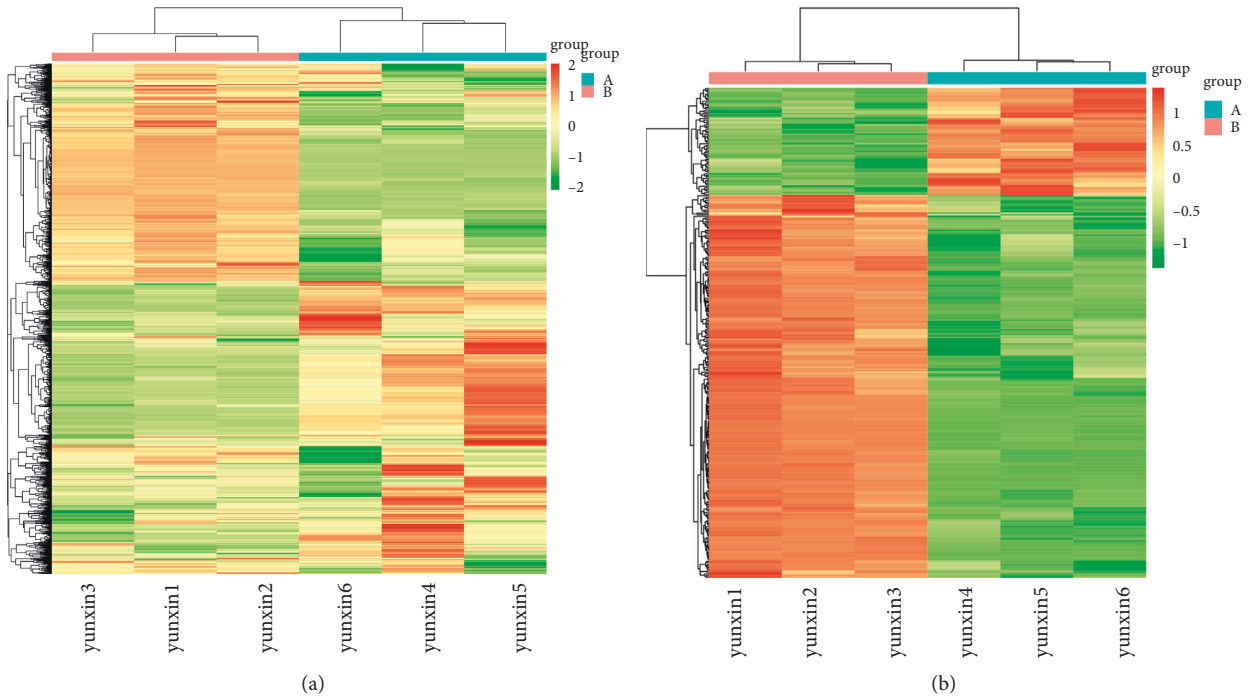

Figure 1: (a) Heatmap visualization of metabolites. (b) Heatmap of DAMs. The content of each metabolite was normalized with complete linkage hierarchical clustering. Each example was visualized in a single column, and each metabolite is represented by a single row. Red indicates high abundance, whereas low relative metabolites are shown in green.

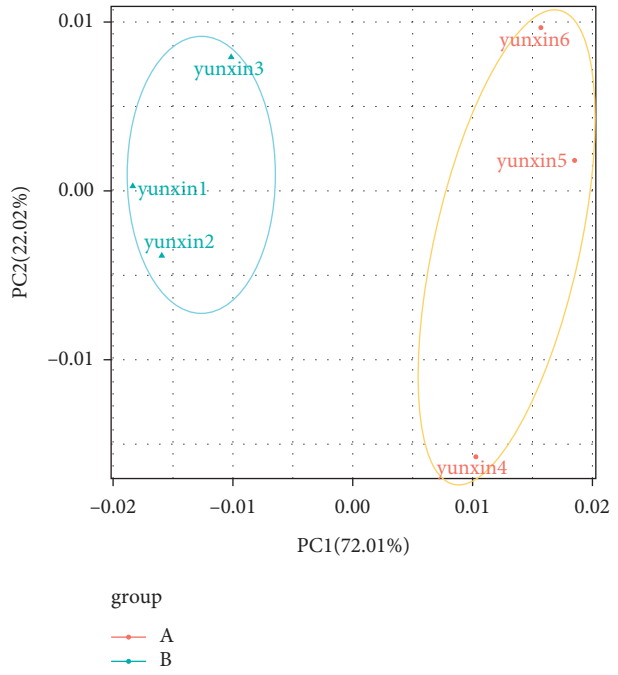

Figure 2: PCA of metabolites.

According to the KEGG pathway enrichment, the phenylpropanoid pathway may have been induced by prochloraz treatment. The phenylpropanoid pathway is an important antidisease pathway of plants; it bifurcates into the production of an enormous array of compounds based on the intermediates of the shikimate pathway in response to cell wall breach by pathogens. The whole metabolomic pathway is a complex network regulated by multiple gene

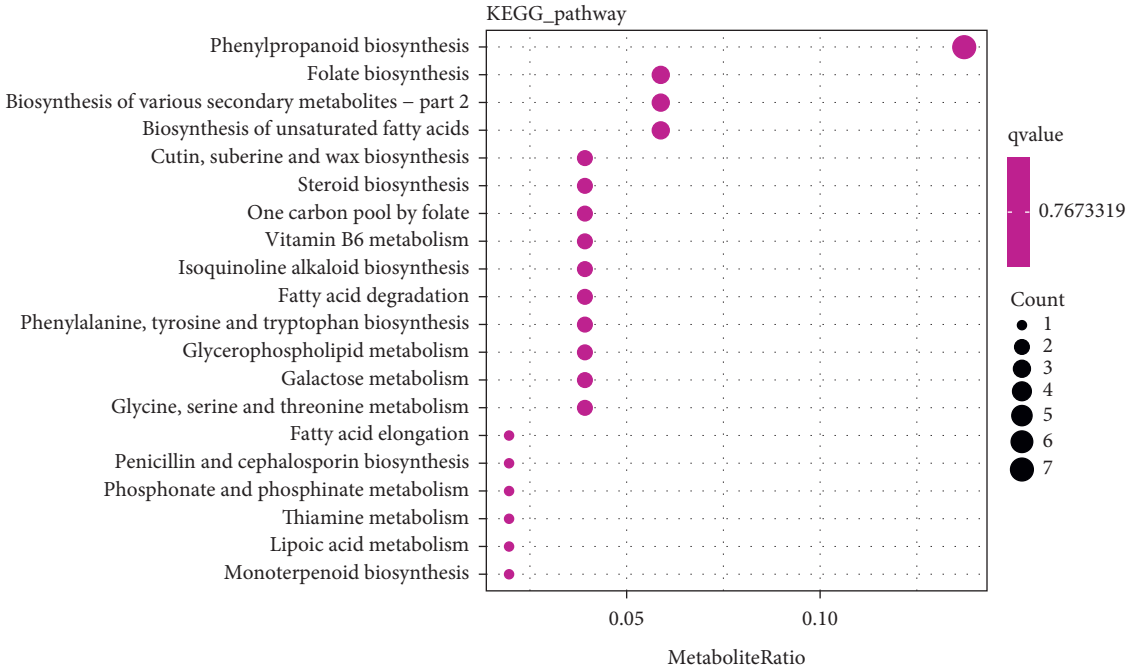

Figure 3: KEGG enrichment pathway of DAMs.

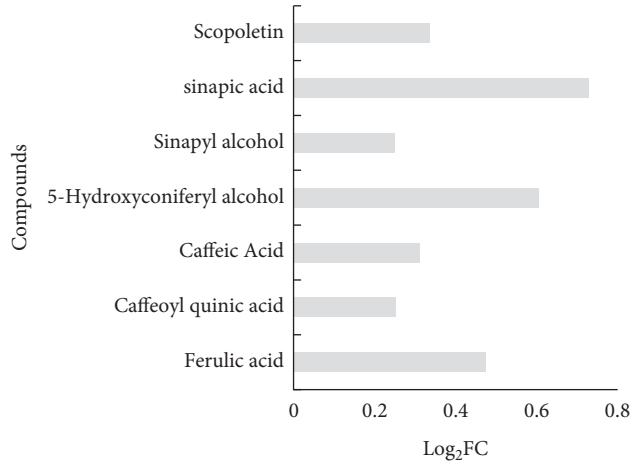

Figure 4: Metabolites of phenylpropanoid biosynthesis.

families, and it exhibits refined regulatory mechanisms at the transcriptional level [19]. The phenylpropanoid is serving as a starting point for the production of many other important compounds, such as flavonoids, coumarins, and lignans [20]; it can regulate downstream metabolite synthesis to respond to pathogen infection. When a pathogen infected plants, the microbe-associated molecular pattern (MAMP)-triggered immunity (PTI) activates a basal defense response, such as the biosynthesis of antimicrobial secondary metabolites or cell wall lignifications, protease inhibitor expression, and hormone biosynthesis [21, 22], and the lignin metabolism in plants regulated defense response that induced by pathogen [23]. According to this research, the content of sinapic acid, caffeoyl quinic acid, caffeic acid, 5-hydroxyconiferyl alcohol, sinapyl alcohol, ferulic acid, and scopoletin increased after treating and may lead to the content of lignin increase to resistant plant pathogen. As known to all, lignin plays an important role in the composition of plant cell walls [24–26]; therefore, prochloraz treatment may induce the synthesis of lignin in the cell wall to resist *C. gloeosporioides* infection expected, inhibiting the synthesis of ergosterol of pathogen.

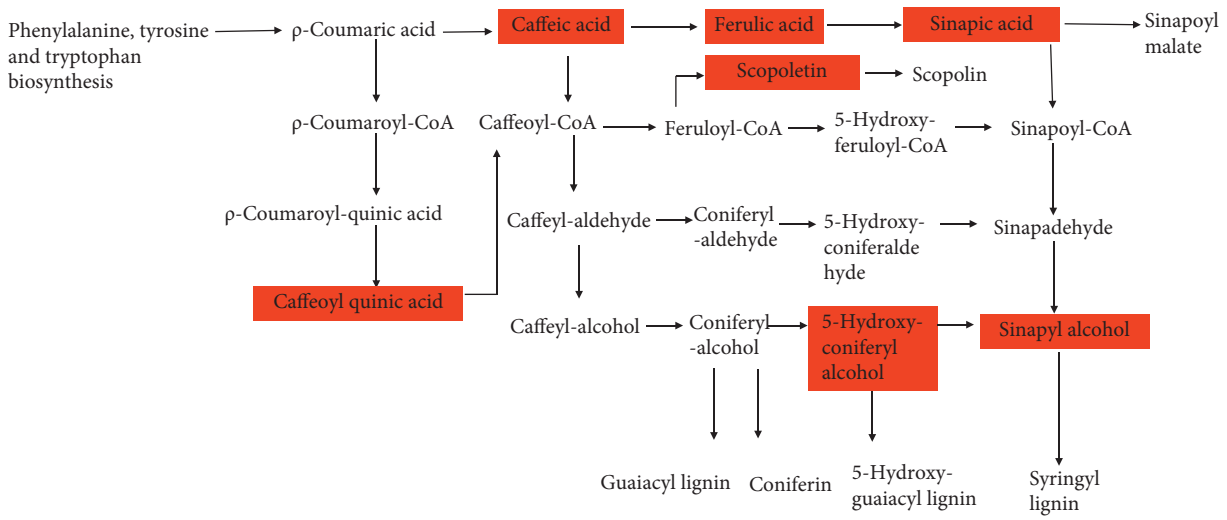

Figure 5: Expression profiles of metabolites involved in phenylpropanoid biosynthesis in CK and PT. The rectangle patterns represent the metabolites for the comparison of CK versus PT. Red indicates upregulation. Green indicates downregulation. White indicates the metabolites that were not annotated.

4. Conclusions

In conclusion, 45% prochloraz EW had the best in vitro and in vivo bioactivities against *C. gloeosporioides* with the EC_{50} values and control efficiency of 0.067 μg/mL and 73.28%, respectively. Meanwhile, metabolome analysis showed that the phenylpropanoid pathway may be induced by prochloraz to increase the metabolites of lignin to resist *C. gloeosporioides* infection.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This work was supported by the Guizhou Province Science and Technology Plan Project Natural Science ((2020) Y129), Guizhou Province Forestry Bureau Young Talent Found ((2019)12), and Guizhou Province Walnut Engineering Technology Research Center ((2019) 5202).

Supplementary Materials

Table S1: all metabolites in samples. Table S2: differentially accumulated metabolites in samples. Figure S1: phenylpropanoid biosynthesis, KEGG pathway. (*Supplementary Materials*)

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