Physiological and metabolic analyses provide insight into soybean seed resistance to *Fusarium fujikuroi* causing seed decay

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Seed-borne pathogens cause diverse diseases at the growth, pre- and post-harvest stage of soybean resulting in a large reduction in yield and quality. The physiological and metabolic aspects of seeds are closely related to their defense against pathogens. Recently, *Fusarium fujikuroi* has been identified as the dominant seed-borne fungi of soybean seed decay, but little information on the responses of soybean seeds induced by *F. fujikuroi* is available. In this study, a time-course symptom development of seed decay was observed after *F. fujikuroi* inoculation through spore suspension soaking. The germination rate and the contents of soluble sugar and soluble protein were significantly altered over time. Both chitinase and β-1,3-glucanase as important fungal cell wall–degrading enzymes of soybean seeds were also rapidly and transiently activated upon the early infection of *F. fujikuroi*. Metabolic profile analysis showed that the metabolites in glycine, serine, and threonine metabolism and tryptophan metabolism were clearly induced by *F. fujikuroi*, but different metabolites were mostly enriched in isoflavone biosynthesis, flavone biosynthesis, and galactose pathways. Interestingly, glycitein and glycitin were dramatically upregulated while daidzein, genistein, genistin, and daidzin were largely downregulated. These results indicate a combination of physiological responses, cell wall–related defense, and the complicated metabolites of soybean seeds contributes to soybean seed resistance against *F. fujikuroi*, which are useful for soybean resistance breeding.

**KEYWORDS**
soybean, seed decay, *F. fujikuroi*, metabolites, metabolic pathway, isoflavone biosynthesis
Introduction

Soybean is one of the important oilseed crops and rich in high-quality vegetable protein, but soybean seed quality and yield are often affected by seed-borne diseases (Wrather et al., 2003). These diseases frequently occur and severely decrease seed quality and reduce seed germination and vigor. Some infected seeds can even act as important carriers to spread important diseases over long distances (Lv and Sun, 2007). Seed decay is one of the most damaging seed-borne diseases at the pre- and post-harvest stage of soybean. In 1985, Phomopsis longicola Hobbs was firstly reported to cause seed decay (Hobbs et al., 1985), and during 1996–2007, this pathogen resulted in soybean yield loss as high as 4.5 million tons in North America (Wrather and Koenning, 2009). Several species in the genus Phomopsis/Diaporthe species complex such as Diaporthe phaseolorum var. caulivora, D. phaseolorum var. meridionalis, and D. phaseolorum var. sojae are also the pathogens of soybean seed decay as well as pod blight (Zhang et al., 1998; Li et al., 2010; Petrovic et al., 2016). Chiotta et al. (2016) found that Fusarium graminearum and F. meridionale were able to infect soybean leading to pod blight and seed decay. More recently, Chang et al. (2020a) reported that F. fujikuroi, F. proliferatum, F. verticillioides, F. asiaticum, and F. incarnatum were the seed-borne pathogens of soybean under the maize–soybean intercropping in Southwest China. Within these Fusarium, F. fujikuroi (teleomorph Gibberella fujikuroi), one member of the polyphyletic taxon in the G. fujikuroi species complex (GFSC), is previously well known as the seed-borne pathogen of rice banaeae diseases (Sun and Snyder, 1981; O’Donnell et al., 1998; Leslie et al., 2005; Summerell et al., 2010) but was later found causing devastating diseases in other economically important plants including sugarcane, wheat, and maize (Cen et al., 2020). In addition, F. fujikuroi produces a variety of secondary metabolites such as fumonisins and gibberellins, which result in serious economic losses and threaten human and livestock health (Suga et al., 2019). To effectively reduce the seed damage caused by F. fujikuroi, it is therefore necessary to elucidate the seed infection mechanism of F. fujikuroi as well as the seed resistance mechanism of its hosts.

During the interaction of pathogens and plants, the preformed physical barriers as well as a range of induced resistance in plant hosts are the important defense lines to defend against pathogens (Dodds and Rathjen, 2010). Seed coat is the key physical barrier of plant seeds to confront diverse pathogens surrounding them, and this layer of resistance not only depends on its structure such as the integrity, thickness, fissures, and cavities of the fenestrated tissue cells but is also closely correlated with diverse chemical compounds produced by the seed coat (Soni et al., 2020). Liang et al. (2000) reported that peanut varieties resistant to Aspergillus flavus were characterized by thicker cell walls, more tightly bound epidermal cells, and denser fenestra layer than disease-susceptible varieties. Once the preformer barriers fail to prevent pathogens, a range of induced resistance are sequentially activated, typically through the enhancement of plant cell walls through callose deposition at infection sites (Chen and Kim, 2009; Luna et al., 2011), the production of reactive oxygen species (ROS) as antifungal and signaling molecules (Torres et al., 2006), and the secretion of other antifungal compounds (Gonzalez-Lamothe et al., 2009). In addition, some pathogenesis-related (PR) proteins such as chitinases and β-1,3-glucanases can be induced to directly target the fungal cell wall structure (Yeoh et al., 2012; Mao et al., 2014), inhibit fungal mycelial growth, and reduce the spore germination of pathogenic fungi (Maher et al., 1993; Egea et al., 1996).

Metabolites often are closely related to seed defense against pathogens (Deng et al., 2017; Li et al., 2021). The phenylpropane pathway is the most important metabolic pathway, and it can upregulate the biosynthesis of secondary metabolites flavonoids, isoflavones, and phenolics, which have been proven as phytoalexins in response to biotic and abiotic stimuli (Steelo et al., 1999; Jung et al., 2000). The biosynthesis of these metabolites can be regulated by several genes including flavanone 3-hydroxylase (F3H), isoflavone synthase (IFS), chalcone synthase (CHS), chalcone isomerase (CHI), dihydroflavonol 4-reductase (DFR), isoflavone reductase (IFR), and anthocyanidin synthase (ANS) (Chen et al., 2019). In addition, some small-molecule metabolites such as terpenoids and alkaloids are rapidly accumulated in large quantities to enhance plant resistance. Liang, as an important component of the secondary wall, maintains the basic life process of living organisms (Miedes et al. 2014). Deng et al. (2017) reported that the biosynthesis and accumulation of alkaloids in the seed coat of resistant soybean germplasm provide high antifungal activity to preharvest field mold caused by the infection of F. verticillioides. Although metabolomics, as an indispensable link between genes, proteins, and phenotypes, has widely been used in the study on plant chemical defense mechanism in the plant–pathogen interactions (Shi et al., 2021), the information on the key defense-related metabolites and corresponding metabolic pathways of soybean seeds in response to seed-borne pathogens is still far uncovered.

In the present study, we tested the physiological and metabolic responses of soybean seed induced by F. fujikuroi after spore suspension soaking at varied time intervals; in particular, the key metabolites and metabolic pathways responsible for seed resistance were explored through metabonomic analysis. It is predicted that this study will provide a useful reference for the better understanding of the seed resistance mechanism and for soybean resistance breeding against seed-borne F. fujikuroi.
Materials and methods

Pathogen inoculation and disease assessment

F. fujikuroi (isolates No. S88) was previously isolated from soybean rotted seeds in Zigong, Sichuan, China, and was identified based on translation elongation factor 1 α (EF-1α) and DNA-directed RNA polymerase II second largest subunit (RPB2) genes (Chang et al., 2020a). This fungus was cultured on potato dextrose agar (PDA, 200 g·l⁻¹ potato, 15 g·l⁻¹ agar and 10 g·l⁻¹ glucose anhydrous) containing 50 μg·ml⁻¹ streptomycin at 25°C for 3 days. For sporulation, a mung bean liquid medium was prepared by boiling 30 g of mung bean in 1 liter of sterilized water for 20 min, filtering the mixture with cheesecloth, and then autoclaving at 121°C for 30 min (Zhou et al., 2016). Five mycelial plugs of F. fujikuroi (5 mm in diameter) were transferred into 30 ml of the mung bean liquid medium in an Erlenmeyer flask and shaken at 25°C, 150 r·min⁻¹ for 4 days to obtain the spore suspension with a concentration of 1 × 10⁸ spores per milliliter.

The soybean seeds of the cultivar Nandou 12, moderately susceptible to F. fujikuroi, were rinsed with running tap water for 20 min, surface-disinfected with 1% NaClO for 3 min, and washed with sterile water three times. After that, the remaining water of the seed surface was finally dried with sterile filter paper. Disinfected seeds were soaked into the spore suspension of F. fujikuroi for 5 min and placed on petri plates containing PDA medium. The seeds soaked in a mung bean soup medium were used as the inoculated controls at 0 day post inoculation, while those seeds soaked in sterilized water for 0–7 days were designed as negative controls as compared to the corresponding inoculated seeds. Treatments at each time points contained 5 plates with 10 seeds on each one, and three independent experiment replicates were conducted. All petri plates were incubated at 25°C in the dark in a constant temperature incubator.

From 0 to 7 days post inoculation of F. fujikuroi, the mycelial coverage area on the seed surface and the rot symptoms inside the seeds were observed. The germination rate, mycelial coverage percentage (MCP), and disease severity index (DSI) were calculated day by day as described by Chang et al. (2020a). The disease grade was evaluated with the range of 0–4 grade as follows: 0 = healthy seed germination without discoloration inside the seeds; 1 = delayed germination with negligible or no discoloration inside the seeds; 2 = low germination with slightly water-soaked and yellow symptoms inside the seeds; 3 = no germination with partially water-soaked, yellow or brown, softened decay inside the seeds; and 4 = no germination, brown, and severe seed decay. The DSI was calculated according to the formula as follows:

\[
\text{DSI} = \frac{\sum (\text{severity grade} \times \text{seeds per grade})}{\text{total seeds} \times \text{the highest severity grade}} \times 100
\]

Determination of the contents of soluble protein and soluble sugar

Soybean seeds were collected from 0 to 7 days post inoculation of F. fujikuroi and used to analyze the contents of soluble protein and soluble sugar. A total of 0.5 g of soybean seeds were rapidly ground with liquid nitrogen into powder and were then transferred into 7 ml of extract solution (1% PVP, 2 mM pH 8.0 EDTA, 0.04% β-mercaptoethanol) in a 10-ml Eppendorf centrifuge tube. After ice bath for 30 min, the extract was centrifuged at 4 000 r·min⁻¹ for 20 min, and then, the supernatant was collected into a tube with a volume scale. The soluble protein content of the supernatants was determined through the Coomassie brilliant blue method as described by Xiong (2003).

The soluble sugar content of soybean seeds was examined using the anthrone colorimetry method (Li, 2002). A total of 0.5 g of well-ground seed powder was homogenated with distilled water to a final volume of 25 ml, and then, 10 ml of the homogenate solution was centrifugated at 4000 r·min⁻¹, 4°C for 3 min. The supernatants were used as the crude enzyme solution and were gradient-diluted with distilled water. The diluted enzyme solution was fully mixed with 2.5 ml of 0.2% anthrone solution, and then the OD₆₅₀ value of the mixture was examined at room temperature using the equal volume of distilled water as a control. Three replicates were tested for each sample. The contents of soluble sugar were calculated by a standard curve method that was prepared based on a standard sucrose solution.

For both soluble sugar and soluble protein, five plates were prepared for each inoculated time points from 0 to 7 dpi, and 10 seeds were placed on each plate. There were three independent replicates.

Activity assay of chitinase and beta-1,3-glucanase

The chitinase activity of soybean seeds after F. fujikuroi inoculation was examined according to Wu et al. (2007). Approximately 0.5 g of well-ground seed powder of soybean was mixed gently with 2 ml of acetic acid extract (0.05 M, pH 5.0), centrifuged at 12000 r·min⁻¹, 4°C for 15 min, and then the supernatants were stored at 4°C as the crude extract enzyme of chitinase. The reaction solution was prepared with 1.5 ml of colloidal chitin solution, 0.5 ml of 0.1 M acetate buffer (pH 4.5), 0.4 ml of enzyme solution, and 0.1 ml of 75 μM sodium azide solution and was then incubated at 37°C for 2–4 h followed by supplementing with 0.5 ml of 0.8 M sodium borate buffer (pH 9.1). After centrifugation at 4000 r·min⁻¹ for 5 min, the supernatants of the reaction solution were collected, and approximately 1.5 ml volume were mixed with chitin.
exonuclease to produce N-acetylglucosamine as treatments. The same volume of the reaction solution was used as a standard control. Finally, 2 ml of potassium perctenate solution was added to the treatment and a standard control, respectively. After keeping in boiling water at 100°C for 15 min, the optical density was read at 420 nm using distilled water as a blank control. The contents of N-acetylglucosamine were calculated by a standard curve method.

The crude enzyme solution of β-1,3-glucanase was prepared as chitinase above. For reaction solution, kombucha polysaccharides preheated at 50°C for 3 min was mixed with the same volume of crude enzyme dilution when mixed with distilled water as blank controls. The reaction mixture was incubated at 50°C for 1 h, supplemented with 2 ml of the DNS reagent, and then rapidly incubated in boiling water bath for 5 min. The reaction was stopped by adding 12 ml of distilled water, and the OD540 values of all reaction samples were measured by zeroing the blank controls. Three replicates were prepared for each sample with at least three independent experiments. A standard curve of glucose solution was prepared as follows:

\[
y = 0.001x - 0.0428, \quad R^2 = 0.9991
\]

**Metabolites analysis using Ultra Performance Liquid Chromatography Tandem Mass Spectrometry**

For metabolic profile analyses, soybean seeds after *F. fujikroi* through the spore suspension soaking method were collected after 0, 3, and 5 days, immediately frozen in liquid nitrogen, and stored at -80°C for further analysis. Each treatment time contained six biological replicates. Approximately 100 mg of seed samples with six biological replicates each treatment were individually grounded with liquid nitrogen into well power. The homogenate was resuspended with precold 80% methanol and 0.1% formic acid, incubated on ice for 5 min, and centrifuged at 15,000 g, 4°C for 20 min. The supernatant was diluted to final concentration using 53% methanol in LC-MS grade water, and the OD540 values of all reaction samples were measured by zeroing the blank controls. Three replicates were prepared for each sample with at least three independent experiments. A standard curve of glucose solution was prepared as follows:

\[
y = 0.001x - 0.0428, \quad R^2 = 0.9991
\]

The same volume of all tested samples was mixed as quality control (QC) samples to balance the state of the LC-MS/MS system and the detection instrument for the evaluation of the system stability. Meanwhile, the blank samples were set to remove background ions.

Ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analyses were performed using a Vanquish UPLC system (Thermo Fisher, Dreieich, Germany) coupled with an Orbitrap Q Exactive™ HF mass spectrometer (Thermo Fisher, Dreieich, Germany) in Novogene Co., Ltd. (Beijing, China). Samples were injected into a Hypesil Gold column (100 × 2.1 mm, 1.9 μm) using a 17-min linear gradient at a flow rate of 0.2 ml-min⁻¹. A Q Exactive™ HF mass spectrometer was operated in both positive and negative polarity mode with a spray voltage of 3.2 kV, a capillary temperature of 320°C, a sheath gas flow rate of 40 arb, and an aux gas flow rate of 10 arb. Eluent A (0.1% formic acid in water) and eluent B (methanol) were used for the positive polarity mode, while eluent A (5 mM ammonium acetate, pH 9.0) and eluent B (methanol) were used for the negative polarity mode. The solvent gradient was set as follows: 2% B for 1.5 min, 2%–100% B for 12.0 min, 100% B for 14.0 min, 100%–2% B for 14.1 min, and 2% B for 17 min.

**Data processing of metabolic profile and metabolite annotation**

The raw data files generated by UPLC-MS/MS were processed using the Compound Discoverer 3.1 (CD3.1, Thermo Fisher, Waltham, MA USA). The peak alignment, peak picking, and quantitation for each metabolite were analyzed. The main parameters were set as follows: a retention time tolerance of 0.2 min, an actual mass tolerance of 5 ppm, a signal intensity tolerance of 30%, a signal/noise ratio of 3:1, and a minimum intensity of 100 000. After that, peak intensities were normalized to the total spectral intensity. The normalized data were used to predict the molecular formula based on additive ions, molecular ion peaks, and fragment ions. Metabolite peaks were then matched with the mzCloud (https://www.mzcloud.org/), mzVault, and MassList databases to obtain the accurate qualitative and relative quantitative results. Statistical analyses were conducted using the statistical software R (R version R-3.4.3), Python (Python 2.7.6 version), and CentOS (CentOS release 6.6). Normal transformations were attempted using the area normalization method when data were not normally distributed.

These metabolites were annotated through the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/pathway.html). Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were conducted at metaX. Univariate analysis (t-test) was applied to calculate the statistical significance (P-value). The metabolites with a variable importance in projection (VIP) score > 1 and p-value< 0.05 and fold change (FC) ≥ 2 or FC ≤ 0.5 were considered to be differential metabolites (DMs). Volcano plots were used to filter the metabolites of interest based on the log2 (fold change) and -log10 (p-value) of metabolites. For clustering heat maps, data were normalized using the z-scores of the
Results

Disease symptoms of soybean seeds after *F. fujikuroi* inoculation

To uncover the time-course symptoms of soybean seeds after *F. fujikuroi* infection, the mycelial growth on the seed surface and the rot symptoms inside the seeds were evaluated. We found that the mycelium of *F. fujikuroi* rapidly grew on the seed surface, and typical symptoms were characterized by water-soaked, brown, and even rot inside the seeds over time when compared with non-inoculated control seeds (Figure 1A). The percentage of mycelium coverage increased sharply after 3 dpi, shortly remained, and continuously reached up to 93.60% at 6 dpi followed by severe seed rot inside (Figure 1B). The DSI had almost the same mycelium coverage but went up the peak at 5 dpi (Figure 1C). In addition, the germination of inoculated seeds was significantly suppressed (p<0.01) and only reached 86% at 7 dpi, whereas the non-inoculated seeds totally germinated at 2 dpi (Figure 1D). Upon *F. fujikuroi* infection, the seed fresh weight significantly increased over the inoculation time and peaked at 6 dpi because of abundant mycelium growing on the seed surface (p<0.05), and they were much higher than those non-inoculated seeds (Figure 1E).

Physicochemical parameters of soybean seeds infected by *F. fujikuroi*

As the important seed responses to pathogen infection, soluble protein and soluble sugar were examined after *F. fujikuroi* infection. It showed that the content of soluble protein gradually increased upon pathogen inoculation, peaked at 3 dpi with the content of 33.71 mg·g⁻¹, and then significantly went down at 5 dpi followed by severe seed rot (Figure 2A). Compared with that at 0 dpi, *F. fujikuroi* infection also caused a rapid decline in the content of soluble sugar until 5 dpi and then slightly increased at 6 dpi in the range of 3.50–18.88 μg·g⁻¹ (Figure 2B). These results showed that the seeds of Nandou produced more soluble protein to defend against pathogens while a significant decrease in the soluble sugar content contributed to the enhanced respiration of soybean.

Chitinase and β-1,3-glucanase are the important cell wall-degrading enzymes targeting chitin and β-1,3-glucan of the fungal cell wall components, respectively. As shown Figures 2C, D, *F. fujikuroi* rapidly activated these two enzymes. Compared to those at 0 dpi, the activities of chitinase in the inoculated seeds rapidly rose up to 106.40 U·g⁻¹ FW at 2 dpi and then kept a slight shift in the range of 58.21–106.40 U·g⁻¹FW. Similarly, β-1,3-glucanase activity dramatically increased 3.0-fold as compared to that at 0 dpi and then gradually decreased over the inoculation time. Thus, the activation of the cell wall-degrading enzymes chitinase and β-1,3-glucanase might contribute for the early defense of soybean seeds against *F. fujikuroi* infection.

Qualification evaluation of metabolic data and seed metabolites annotated in response to *F. fujikuroi* inoculation

According to PCA, metabolic profiles at three time intervals postinoculation of *F. fujikuroi* were clearly distributed into three separate groups, in which the first two principal components explained 75.41% of the variability, containing 58.91% [principal component 1 (PC1)] and 16.50% [principal component 2 (PC2)], respectively (Figure 3). All six biological replicates at each time interval were clustered together, indicating that little difference exists among biological replicates and the quality of data are very high for further metabolic profile analyses.

According to KEGG pathway annotation, the seed metabolites of soybean induced by *F. fujikuroi* were the most highly enriched in the global and overview maps. There were more metabolites enriched in the biosynthesis of other secondary metabolites, amino acid metabolism, carbohydrate metabolism, the metabolism of cofactors and vitamins, and lipid metabolism than other metabolites (Figure 4A). Annotation results from the Human Metabolome Database (HMDB) database showed that lipids and lipid-like molecules were the most enriched metabolites of soybean induced by *F. fujikuroi* (Figure 4B). Furthermore, the annotation using LIPID MAPS showed that 42 metabolites in the flavonoid pathway were mostly enriched when sterols, isoprenoids, fatty acids, and conjugates also had relatively high accumulation as compared to other metabolites (Figure 4C).
Symptoms of seed rot caused by *F. fujikuroi* and related seed parameters. (A) Time-course symptoms of soybean seeds with or without *F. fujikuroi* inoculation were observed over time. (B) Percentage of mycelium coverage was calculated by the coverage area of mycelium on the seed surface. (C) Disease severity index (DSI) was evaluated and calculated according to disease symptoms. Germination rate (D) and seed fresh weight (E) of the non-inoculated and inoculated seeds with *F. fujikuroi* were recorded over the inoculation time. Statistical analysis was conducted using Statistical Product and Service Solutions (SPSS) version 23.0 with the ANOVA method; ** indicates significant difference at the level of \( p = 0.01 \), while * stands for significant difference at the level of \( p = 0.05 \). Each treatment contains five plates with 10 seeds on each plate \( (n = 50) \), and three independent experiments were conducted.
Quantitative analysis of different metabolites of soybean seeds induced by *F. fujikuroi*

To uncover the key seed metabolites responding to *F. fujikuroi*, different metabolites were analyzed among three comparison groups of S88_3 vs S88_0, S88_5 vs S88_3, and S88_5 vs S88_0 over inoculation time. PCA loading plots showed a significant change in different metabolites in each comparison group (Figures 5A1, B1, C1), among which the S88_3 vs S88_5 group displayed the most diffuse distribution (Figure 5B1). For the PLS-DA analysis, three comparison groups were scored 82.82%, 65.51%, and 86.08% in PC1, respectively (Figures 5A2, B2, C2). After sevenfold cross-validation, the R² and Q² values in each comparison group were all close to 1 (Figures 5A3, B3, C3), implying that the predictive ability and quality of the three groups of models are suitable for subsequent experiments. Thus, based on the PCA and PLS-DA analysis, *F. fujikuroi* induced the significant time-course changes in the metabolites of soybean seeds.

It is clearly seen that *F. fujikuroi* infection remarkably induced the different patterns of upregulated and downregulated metabolites among three comparison groups (Figure 6A). There were the most upregulated metabolites such as coumestrol, kynurenic acid-O-hexside, gramine, and isorhapontigenin in the S88_3 vs S88_0 group, when more downregulated metabolites including desthiobiotin, glycitein, glycyl-L-leucine, and 8,8-dimethyl-2H,8H-pyrano [3,2-g] chromen-2-one, 4’7-dihydroxyflavanone, were accumulated rather than upregulated ones in the S88_5 vs S88_3 group (Figures 6B, C). In the S88_5 vs S88_0 group, the top five different metabolites included pantethenic acid, gamma-glutamylleucine, 3-hydroxy-4-methoxy-9H-xanthene-9-one,
gamma-glutamyltyrosine, and acetyl-trans-resveratrol (Figure 6C), but there was no significant difference in the numbers of upregulated and downregulated metabolites (Figure 6B).

**Different KEGG pathways of soybean seeds activated by *F. fujikuroi* over time**

To further uncover the metabolic pathways participating in soybean seed defense against *F. fujikuroi*, the KEGG annotation of seed metabolites was analyzed (Supplementary Table S1). In the S88_3.vs.S88_0 group, we found that there are 143 differential metabolites enriched in 35 metabolic pathways. Among them, glycine, serine, and threonine metabolism with 5 different metabolites including L-homoserine, betaine aldehyde, L-tryptophan, L-cystathionine, and betaine, was the most enriched metabolic pathway in response to *F. fujikuroi* infection (Figure 7A). In addition, *F. fujikuroi* also activated other metabolic pathways such as biotin metabolism, tropane, piperidine and pyridine alkaloid biosynthesis, porphyrin and chlorophyll metabolism, cysteine and methionine metabolism, and arginine and proline metabolism. In the S88_5.vs.S88_3 group, 65 differential metabolites were enriched in 28 metabolic pathways, and the most relevant metabolic pathway to *F. fujikuroi* infection was isoflavonoid biosynthesis (identified metabolites: formononetin, daidzein, glycitin, glycine, biochanin A, and genistein), galactose metabolism (identified metabolites: inositol and d-sorbitol), and caffeine metabolism (identified metabolites: 1-methyluric acid and xanthine) (Figure 7B). Specifically, the five pathways of galactose metabolism, ABC transporters, fructose and mannose metabolism, tryptophan metabolism and beta-alanine metabolism were overlapped between the S88_3.vs.S88_0 group and the S88_5.vs.S88_3 group. In the S88_5.vs.S88_0 group, a total of 195 differential metabolites were enriched in 39 metabolic pathways; the most relevant metabolic pathway in response to *F. fujikuroi* was glycine, serine, and threonine metabolism involving six metabolites: l-homoserine, betaine aldehyde, l-tryptophan, choline, l-cystathionine, and betaine (Figure 7C). Hence, according to the pathway enrichment analysis, flavonoids could be involved in the *F. fujikuroi*-treated seed defense against *F. fujikuroi*.

**FIGURE 3**
PCA score of different metabolites and the correlation analysis of QC samples. The three samples under three inoculation time points of *F. fujikuroi* were named as S88_0, S88_3, and S88_5, respectively. The six biological replicates corresponding to each sample were named S88_0_a, S88_3_a, and S88_5_a, respectively, where a ∈ {1, 2, 3, 4, 5, 6}.
Top 20 different metabolites of soybean seeds induced by *F. fujikuroi*

According to the analysis of the key different metabolites of soybean seeds induced by *F. fujikuroi* over time, the top 20 different metabolites in each comparison group were screened based on the p-value and FC (Table 1). Our results showed that in the S88_3.vs.S88_0 group, the main different metabolites included isorhapontigenin, coumestrol, 7-hydroxy-3-(4-methoxyphenyl)-4h-chromen-4-one, s-adenosyl-l-homocysteine, myricetin, glycitein, coniferin, and others, and 17 of the top 20 metabolites were upregulated upon *F. fujikuroi* inoculation. In contrast, only columbianetin acetate and nor-9-carboxy-9-thc were upregulated in the S88_5.vs.S88_3 group, whereas other metabolites such as glycine, glycyll-leucine, desethylbiotin, 4,7-dihydroxyflavanone, 6′-o-acetylglucin, glycitin, genistein, phloridzin, formononetin, and daidzin, were significantly downregulated. In addition, the different metabolites in the S88_5-vs-S88_0 group mainly included γ-glutamylytyrosine, γ-glutamylyleucine, 6-methylquinoline, eriodictyol-7-o-glucoside, dl-tryptophan, 1-cystathionine, and menadione. Among them, the different metabolites such as n-(1,3-benzodioxol-5-yl)-2-methyl-5-(piperidinosulfonyl)-3-furamide, n-(4-chlorophenethyl)-n′-(4-chlorophenyl)urea, coumestrol and kynurenic acid o-hexside overlapped in the S88_3-vs-S88_0 group and S88_5-vs-S88_0 group. Except for coumestrol which is upregulated in S88_3-vs-S88_0 group, the other three metabolites were also significantly downregulated. Three metabolites including 6-methylquinoline, dl-tryptophan, and...
γ-glutamyltyrosine were also the overlapping differential metabolites in the S88_5.vs.S88_3 group and S88_5.vs.S88_0 group, and they were all significantly downregulated at the same time (Table 1).

**F. fujikuroi** activated the isoflavonoid metabolites in soybean seeds

Since the isoflavonoid pathway was significantly activated upon *F. fujikuroi* infection, and several metabolites in this pathway showed remarkable changes as the top 20 metabolites, a simple metabolic pathway was drawn to clearly display the change pattern of the key metabolites in this pathway. As shown in Figure 8, two upstream metabolites, naringenin and liquiritigenin, were transiently upregulated at 3 dpi and then decreased at 5 dpi. Their downstream metabolites such as genistein, glycitein, and daidzein, showed distinct change patterns. As the downstream of naringenin, genistein was not detectable at 3 dpi but significantly downregulated at 5 dpi. Among three downstream metabolites of genistein, biochanin A and genistin showed the similar change, but 2'-hydroxygenistein

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**FIGURE 5**
Statistical analysis for metabolite differences among three comparison groups after *F. fujikuroi* infection. Metabolic data of soybean seeds induced by *F. fujikuroi* were statistically analyzed among three comparison groups including S88_3.vs.S88_0, S88_5.vs.S88_3, and S88_5.vs.S88_0. (A1–A3) PCA loading plots. (B1–B3) PLS-DA score plots. (C1–C3) PLS-DA valid plots.
was significantly upregulated at 3 dpi. For the downstream metabolites of liquiritigenin, glycitein and its glycitin were significantly and transiently accumulated at 3 dpi and then rapidly decreased, indicating their role in soybean seed resistance to *F. fujikuroi* infection. Additionally, daidzein acts as a central component of the isoflavonoid biosynthetic pathway and the precursor of the soybean phytoalexins glyceollins and coumestrol, and in our results, daidzein was remarkably downregulated at 3 dpi upon *F. fujikuroi* infection and its two downstream metabolites formononetin and daizin were sequentially downregulated at 5 dpi. Moreover, the soybean phytoalexin coumestrol related to daidzein synthesis was rapidly accumulated at 3 dpi, indicating that the accumulation of coumestrol in soybean seeds tissues is the signal of early defense response. Thus, the accumulation of metabolites in the isoflavone biosynthesis pathway contributes to soybean seed resistance to *F. fujikuroi*.

**Discussion**

Soybean seed-borne diseases can be caused by several pathogens including the *Phomopsis/Diaporthe* species complex (Hobbs et al., 1985; Petrović et al., 2016), *Fusarium* spp. (Chiotta et al., 2016; Chang et al., 2020a; Chang et al., 2020b) and *Cercospora* spp. (Li et al., 2019) and have been becoming more and more severe with the continuous increase of the soybean planting area and harsh climate at the preharvest period of soybean (Deng et al., 2017; Li et al., 2019). Previously, many efforts have been made to identify the resistance of soybean resources to *Phomopsis* sp. and *Cercospora* sp. (Li et al., 2015; Li et al., 2017; Li et al., 2019), and a range of soybean lines were reported to have certain levels of resistance. However, this study focused on the interaction of soybean and *F. fujikuroi* and on exploring the physiological and metabolic mechanism of soybean seeds to the seed-borne *F. fujikuroi*. 
The infection of pathogens often activates a range of the physiological and chemical changes in plant hosts. Seed germination is one of the important evaluation factors of seed quality. Our results showed that *F. fujikuroi* remarkably delayed seed germination until 5 days, which is consistent with previous studies in which inoculation with *P. longicolla* reduced the germination of several soybean lines (Li et al., 2015; Li et al., 2017). In addition, several *Fusarium* spp. were also reported to inhibit seed germination (Purahong et al., 2012; Jiang et al., 2020). Soluble sugars and soluble proteins are the important energy substances for plants, and they can provide energy not only for defense substances like PR proteins in order to defend against pathogens.

It is well known that the expression of PR proteins (i.e., chitinases and β-1, 3-glucanases) is often induced as the important aspects of induced systemic resistance during and/or after pathogen attack (Durrant and Dong, 2004; Jain and Khurana, 2018). Chitinases are the enzymes involved in the

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**Figure 7**
Top 20 KEGG pathways enriched in different comparison groups over the *F. fujikuroi* inoculation time. (A–C) are the scatter plots of the enrichment statistics of the KEGG pathway in S88_3 vs S88_0, S88_5 vs S88_3, and S88_5 vs S88_0, respectively. The abscissa in the figure is x/y (the rate of the number of differential metabolites in the corresponding metabolic pathway to the total number of metabolites identified in the pathway), and the larger the values indicate, the higher the enrichment of differential metabolites in the pathway. The color of the dot represents the p-value of the hypergeometric test, and the smaller the values stand for, the greater the reliability of the test and the more statistically significant. The size of the dot represents the number of different metabolites in the corresponding pathway. The larger the dot indicates the more differential metabolites in the pathway.
| Comparison time group | Metabolites | FC       | p-value    | Up- or Down-regulated |
|-----------------------|-------------|----------|------------|-----------------------|
| S88_3.vs.S88_0        | N-(1,3-benzodioxol-5-yl)-2-methyl-5-((piperidinosulfonyl)-3-furamide | 0.02 | $1.3\times10^{-20}$ | down |
|                       | Isoflavonoids | 52.0 | $2.3\times10^{-19}$ | up |
|                       | Gramine      | 26.6 | $3.4\times10^{-19}$ | up |
|                       | Coumestrol   | 53.6 | $1.5\times10^{-18}$ | up |
|                       | Kynurenic acid O-hexide | 20.2 | $5.1\times10^{-18}$ | up |
|                       | 7-hydroxy-3-(4-methoxyphenyl)-4H-chromen-4-one | 149.1 | $1.1\times10^{-17}$ | up |
|                       | S-Adenosyl-L-homocysteine | 104.8 | $2.8\times10^{-17}$ | up |
|                       | Myricetin    | 26.5 | $5.8\times10^{-17}$ | up |
|                       | LysoPC 12:1  | 26.5 | $6.2\times10^{-17}$ | up |
|                       | 7-(3,4-dihydroxyphenyl)-5-hydroxy-1-(4-hydroxyphenyl)heptan-3-one | 27.3 | $2.0\times10^{-16}$ | up |
|                       | Gramine      | 16.6 | $2.5\times10^{-16}$ | up |
|                       | 3-methyl-5-oxo-5-(4-toluidino)pentanoic acid | 18.7 | $6.8\times10^{-16}$ | up |
|                       | IAA-Asp      | 37.1 | $9.7\times10^{-16}$ | up |
|                       | 2-(4-[(trifluoromethyl)thio]phenoxy)ethanohydrazide | 0.2 | $1.1\times10^{-15}$ | down |
|                       | L-Glutamic acid | 2.9 | $1.4\times10^{-15}$ | up |
|                       | Acetyl-trans-resveratrol | 98.5 | $2.5\times10^{-15}$ | up |
|                       | Ethyl-5-[(2,1,3-benzoxadiazol-4-ylsulfonyl)amino]-2-piperidinobenzoate | 0.01 | $3.1\times10^{-15}$ | down |
|                       | 9-HpOTre      | 14.6 | $3.2\times10^{-15}$ | up |
|                       | Coniferin    | 24.5 | $3.6\times10^{-15}$ | up |
| S88_5.vs.S88_3         | 8,8-dimethyl-2H-L8H-pyrano[3,2-g]chromen-2-one | 0.08 | $5.0\times10^{-10}$ | down |
|                       | Glycitein    | 0.17 | $2.3\times10^{-16}$ | down |
|                       | Desthiobiotin| 0.15 | $3.4\times10^{-16}$ | down |
|                       | Coumestrol   | 0.18 | $1.1\times10^{-13}$ | down |
|                       | 4’7-Dihydroxyflavanone | 0.23 | $2.0\times10^{-13}$ | down |
|                       | 6’-O-Acetylglycin | 0.09 | $2.6\times10^{-13}$ | down |
|                       | Glycitin     | 0.08 | $2.8\times10^{-13}$ | down |
|                       | 6-Methylquinoline | 0.52 | $3.3\times10^{-13}$ | down |
|                       | gamma-Glutamyhydrine | 0.26 | $5.8\times10^{-13}$ | down |
|                       | 5,7-dihydroxy-3-(4-hydroxyphenyl)-4H-chromen-4-one | 0.45 | $8.1\times10^{-13}$ | down |
|                       | Genistein    | 0.45 | $8.2\times10^{-13}$ | down |
|                       | Uric acid    | 0.25 | $8.5\times10^{-13}$ | down |
|                       | columbianetin acetate | 6.46 | $2.1\times10^{-12}$ | up |
|                       | DL-Tryptophan | 0.52 | $2.8\times10^{-12}$ | down |
|                       | ABL 127      | 0.11 | $2.8\times10^{-12}$ | down |
|                       | Phlorizin     | 0.19 | $3.0\times10^{-12}$ | down |
|                       | Formononetin | 0.26 | $4.9\times10^{-12}$ | down |
|                       | Linoleic acid | 0.46 | $5.8\times10^{-12}$ | down |
|                       | Nor-9-carboxy-8-THC | 6.41 | $6.2\times10^{-12}$ | up |
|                       | Daidzin      | 0.38 | $1.0\times10^{-11}$ | down |
| S88_5.vs.S88_0         | gamma-Glutamyhydrine | 0.05 | $5.5\times10^{-10}$ | down |
|                       | 3-hydroxy-4-methoxy-9H-xanthen-9-one | 0.07 | $3.5\times10^{-10}$ | down |
|                       | Acetyl-trans-resveratrol | 95.18 | $5.5\times10^{-10}$ | up |
|                       | Isocryptotanshinone | 26.12 | $7.0\times10^{-10}$ | up |
|                       | Pantothenic acid | 0.13 | $2.0\times10^{-10}$ | down |
|                       | 4-(3,4-dihydro-2H-1,5-benzodioxepin-7-ylamino)-4-oxobutanoic acid | 80.68 | $2.1\times10^{-10}$ | up |
|                       | Ste-Dihydrotestosterone | 8.88 | $2.1\times10^{-10}$ | up |
|                       | Kynurenic acid O-hexide | 32.14 | $2.2\times10^{-10}$ | up |
breaking of the β-1,4-glycosidic linkages of chitin, and they are present in the cotyledons and seed coats of soybean acting as an important seed defense molecule targeting the pathogen cell wall (Gijzen et al., 2001). Yang et al. (2020) reported that the overexpression of the chitinase gene CmCH1 enhanced soybean resistance to Sclerotinia sclerotiorum.

β-1,3-glucanases also belong to the PR-2 family of PR proteins, and they often defend plants against fungal pathogens by degrading the fungal cell wall either alone or in association with chitinase (Balasubramanian et al., 2012). In our study, both chitinase and β-1,3 glucanase were rapidly activated in soybean seeds at the early infection of F. fujikuroi, which might contribute for early seed coat resistance to F. fujikuroi infection. To consolidate the pathogenicity, some pathogens have evolved to protein inhibitors to directly inhibit the activities of PRs proteins, such as β-1, 3-glucanases (Rose et al., 2002; Naumann et al., 2009; Sánchez-Rangel et al., 2012). This can explain that the activities of β-1, 3-glucanases gradually decreased after 2 days of F. fujikuroi inoculation. Thus, PRs proteins, typically chitinase and β-1, 3-glucanases, can be induced in soybean seeds to defend against F. fujikuroi infection.

Plants often need to reprogram their primary metabolism to provide energy and build defenses to cope with adverse environmental stresses (Bolton, 2009; Rojas et al., 2014). In this study, the amino acid metabolism was strongly highlighted among the major metabolic pathways reprogrammed upon F. fujikuroi infection (Supplementary Table S2). Many studies have demonstrated that the mobilized amino acid pool provides

### TABLE 1 Continued

| Comparison time group | Metabolites                                                                 | FC            | p-value        | Up- or Down-regulated |
|-----------------------|------------------------------------------------------------------------------|---------------|----------------|-----------------------|
|                       | N-(1,3-benzodioxol-5-yl)-2-methyl-5-[(piperidinosulfonyl)-3-furamide          | 0.03          | 2.9x10^-17     | down                  |
|                       | 6-Methylquinoline                                                           | 0.22          | 3.1x10^-17     | down                  |
|                       | 4-(2,3-dihydro-1,4-benzodioxin-6-yl)-1,2-diphenylbut-2-ene-1,4-dione         | 0.05          | 6.6x10^-17     | down                  |
|                       | Erindectyl-7-O-glucoside                                                    | 0.10          | 2.3x10^-16     | down                  |
|                       | N-buty1-2-methyl-5-[(piperidinosulfonyl)-3-furamide                          | 29.03         | 2.8x10^-18     | up                    |
|                       | DL-Tryptophan                                                               | 0.22          | 5.6x10^-18     | down                  |
|                       | L-Cystathionine                                                             | 27.77         | 7.3x10^-18     | up                    |
|                       | N-(4-chlorophenethyl)-N'-(4-chlorophenyl)urea                               | 44.01         | 8.8x10^-18     | up                    |
|                       | 2-(3,5-dichlorophenyl)-6-methyl-2,3,4,5-tetrahydropyridazin-3-one            | 95.58         | 1.1x10^-15     | up                    |
|                       | L(-)-Carnitine                                                              | 21.49         | 1.7x10^-15     | up                    |
|                       | Menadione                                                                   | 0.11          | 1.8x10^-15     | down                  |
|                       | 3-Methoxy prostaglandin F1st                                                | 57.52         | 1.8x10^-15     | up                    |

FC refers to the difference multiple, which is the ratio of the mean values of all biological repeated quantitative values of each metabolite in the comparison group. The P-value is calculated by the t-test, which indicates the difference significance level. VIP refers to the variable projection importance of the first principal component, which indicates the contribution of metabolites to the subgroup. Up indicates upregulation; down indicates downregulation. The thresholds are set as VIP > 1.0, FC > 1.5 or FC < 0.667 and p-value < 0.05, and the different metabolites.
precursors and even energy for the synthesis of a large number of metabolites that are associated with a response to diverse environment stimuli (Hofmann et al., 2010; Deng et al., 2022). Thus, the activation of the amino acid metabolism might contribute to precursors and energy for other metabolites to adapt or defend against F. fujikuroi. In addition, we found that the glycine, serine, and threonine metabolic, as well as tryptophan metabolism, were the most metabolic pathways related to F. fujikuroi infection at 3 dpi. Previous studies have shown that tryptophan has an important role in plant defense (Chennupati et al., 2012; Qin et al., 2016; Qin et al., 2017). For example, isoavone accumulation enhanced soybean resistance to the mosaic virus (Hao et al., 2007), cyst nematode (Lin et al., 2016), and field mold (Deng et al., 2017). In our study, the inoculation of F. fujikuroi remarkably affected the metabolic profile of soybean seeds and significantly activated the isoflavone pathways and related metabolites. Meanwhile, as the downstream of naringenin and liquiritigenin in the flavonoid pathway, several key metabolites such as genistein, glycitein, daidzein, and genistein were detected to change in different ways upon F. fujikuroi infection. Previous studies demonstrated that naringenin is a potential biomarker of resistance to Fusarium head blight in some wheat and barley varieties (Gunnahiah et al., 2012) and it has also been used as a potent inhibitor of F. graminearum in vitro (Bollina et al., 2010). Deng et al. (2022) showed a significant enrichment of two isoflavone aglycones, genistein and daidzein, in soybean seeds in response to field mold. Morkunas et al. (2010) reported that genistein and genistin were induced to be upregulated at 48 hpi and significantly decreased at 72 hpi using non-inoculated yellow lupine seeds as control, indicating that the two metabolites function at an early infection stage of F. oxysporum shortly after the embryonic axis breaks through the seed coat. However, in our study, regarding the seed disease symptoms, isoflavones were not detected at much earlier stage before 3 days of pathogen inoculation; for example, glycitein was only detected to sharply accumulate at 3 dpi to promote soybean seed resistance to F. fujikuroi. Meanwhile, in our study, we only compared the metabolite difference of soybean seeds among the different inoculated times of F. fujikuroi but did not employ the non-inoculated seeds as control, thus, certain different metabolites might be seed germination and development. Therefore, in further work, the different metabolites upon F. fujikuroi inoculation over time need to be verified by high performance liquid chromatography (HPLC) analysis or the gene expression related to metabolite biosynthesis and regulation.

The plant hormone signaling molecules such as jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA), are usually associated with the defense mechanism of plants against necrotrophic pathogens (Glazebrook, 2005; Spoel et al., 2007; Denance et al., 2013), like the pathogen F. fujikuroi (Matic et al., 2016). In our study, the plant hormone signal transduction pathway was significantly enriched in the scatter plots of the top 20 KEGG pathway at 3 dpi compared to control (S88_3.vs.S88_0). Moreover, Hormonal signaling molecules such as JA, ABA, and auxin, were all enriched, implying a crosstalk of plant hormone signaling is activated to defend against F. fujikuroi (Supplementary Figure S1). Previous studies demonstrated that auxin can act as positive or negative regulators of plant defense (Chen et al., 2007; Llorente et al., 2008; Denance et al., 2013). In the present study, IAA (Indole-3-acetic acid) was transiently upregulated at 3 dpi but sequentially downregulated, which may be employed by F. fujikuroi at early infection stage but latterly repressed to increase soybean susceptibility to F. fujikuroi. In addition, ABA often functions on seed germination and water stress management related to stomata closure in the epidermis (Ali and Baloch, 2020), but it also has different roles at different stages of pathogen infection, in particular, favoring resistance before invasion (Melotto et al., 2006) and susceptibility at later stages of colonization (López et al., 2008). In our study, compared with 0 dpi, ABA was only remarkably upregulated at 3 dpi, probably triggering the stomata closure of inoculated seeds to prevent F. fujikuroi penetration. In another study, Siewers et al. (2004) reported that ABA played a negative role in plant resistance to B. cinerea when interacted with other hormone signaling molecules such as JA or ET. B. cinerea. In this study, JA signaling was clearly downregulated at 3 dpi; however, whether it is related to upregulated ABA signaling needs to be further elucidated in the following work. Overall, F. fujikuroi activated a complicated plant hormone signaling net of soybean seeds, and the crosstalk of these hormones might contribute to seed resistance.

Conclusions

This study, for the first time, demonstrated the physiological and metabolic responses of soybean seeds contributing to its resistance to F. fujikuroi in an integrated and complicated way.
The seed-borne *F. fujikuroi* severely affected the germination of soybean seeds and changed the contents of soluble sugar and soluble protein in a different way. In addition, *F. fujikuroi* also transiently activated two cell wall–degrading enzymes, chitinase and β-1,3-glucanase, at the early infection stage. A large number of metabolites in glycine, serine, and threonine metabolism and tryptophan metabolism were significantly accumulated, while the metabolites involved in isoflavone biosynthesis and flavonoid biosynthesis were differently induced upon *F. fujikuroi* infection over time. These results are helpful to expand the understanding of the interaction mechanism of soybean and seed-borne pathogens as well as the resistance breeding of soybean.

**Data availability statement**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

**Author contributions**

XC, XL, and HL designed the research. XL, HM, and HL performed the research. XW, HC, and XL analyzed the data. XC and XW wrote the paper. CY, GG, and TL edited the paper. MZ, WC, and WY supervised and funded the research. All authors contributed to the article and approved the submitted version.

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**Conflict of interest**

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

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**Supplementary material**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.993519/full#supplementary-material

**SUPPLEMENTARY FIGURE S1**

Plant hormone signal transduction pathway at 3 dpi.

**SUPPLEMENTARY TABLE S1**

KEGG Pathway enrichment results of differential metabolites at three comparison groups over time after *F. fujikuroi*.

**SUPPLEMENTARY TABLE S2**

Changes in metabolites of amino acid metabolic pathways at 3 dpi.

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