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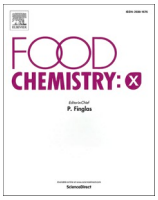

Screening of soybean antifungal isoflavones based on targeted metabolomics analysis

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ABSTRACT

Soybean (*Glycine* max) is a key crop rich in bioactive compounds, particularly isoflavones, which play a significant role in plant defense against biotic stress like fungal infections. In this study, nine soybean varieties with varying susceptibility to mildew were evaluated. Isoflavone profiles in seeds and pods were analyzed using high-performance liquid chromatography-mass spectrometry (HPLC-MS), and metabolomics analysis via orthogonal signal correction partial least squares discriminant analysis (OSC-PLS-DA) identified differences between mildew-affected and unaffected samples. Results showed organ-specific changes, with isoflavone aglycones increasing in seeds, while malonylglucosides (M-type) varied in pods. β-Glucoside (G-type) and M-type isoflavones were identified as differential metabolites. Antifungal assays revealed that genistin, among six isoflavone glycosides and aglycones tested, had the strongest inhibitory effects on *Aspergillus flavus*. Additionally, the identification of G-type and M-type isoflavone glycosides underscores the necessity for further investigation into the roles these metabolites play in the overall antifungal activity observed.

1. Introduction

Soybean (*Glycine* max), domesticated from *Glycine soja* Sieb & Zucc in East Asia, is the leading global source of plant-based protein and oil, playing a crucial role in human nutrition and animal feed (Commission, 2020; Vogel et al., 2021). Numerous clinical studies highpoint the health benefits of soybean consumption, including reductions in low-density lipoprotein cholesterol and blood pressure compared to other legumes (Jenkins et al., 2010; Taku et al., 2010). Diets rich in soybean are also associated with a reduced incidence of cardiovascular diseases such as heart attacks and strokes, attributed to the presence of bioactive compounds like isoflavones, anthocyanins, phenolic acids, and fatty acids (Bouchenak & Lamri-Senhadji, 2013; D'Adamo & Sahin, 2014; Yan, Zhang, Li, Jiao, & Dong, 2017).

China is one of the largest soybean producers globally, with the southwestern region contributing significantly to its production. In Southwest China, the high autumn precipitation about 25 % of annual rainfall creates favorable conditions for soybean cultivation (Liu, Chen, Lian, Chen, & Chen, 2015; Yang et al., 2014). However, the continuous rainfall during the harvest season increases the risk of field mold (FM), caused by fungal pathogens that thrive in humid environments, leading to seed deterioration and substantial yield losses. FM can reduce yields by up to 90 % and degrade seed quality due to diminished storage reserves (Liu et al., 2017). Soybean varieties with dark seed coats (black or brown) show higher resistance to FM stress compared to those with yellow or green seed coats, likely due to the higher concentration of phenolic compounds such as anthocyanins (Deng et al., 2017; Deng JunCai et al., 2015; Liu et al., 2017). Isoflavones, another class of phenolic compounds, are also implicated in soybean resistance to FM stress (Deng et al., 2019).

Globally, mildew remains a significant threat to soybean production, leading to reduced yields and seed quality, particularly in susceptible varieties (Bui et al., 2023). Mildew, which can occur in the field or during storage, is heavily influenced by environmental conditions.

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Pathogenic fungi, such as *Aspergillus flavus* and *Aspergillus parasiticus*, are especially concerning due to their ability to produce aflatoxin B1 (AFB1), a potent carcinogen classified by the World Health Organization (WHO) as a Group 1 carcinogen in 1993. AFB1 contamination poses serious risks to both human and livestock health, compromising the safety and edibility of soybean and its derived products. Soybean is vulnerable to various pathogenic fungi that affect its yield and quality. Studies has identified four primary fungi associated with moldy soybean seeds; *Aspergillus flavus*, *Aspergillus niger*, *Fusarium moniliforme*, and *Penicillium chrysogenum* using both microscopic and molecular biology techniques (Liu et al., 2017). Among these, *Aspergillus flavus* is the most severe contaminant due to its ability to produce aflatoxins such as AFB1, which significantly reduces the nutritional value of soybean by decreasing protein, fat, and soluble protein content (Liu et al., 2017). Aflatoxin production is primarily associated with the Aspergillus species *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* (Benkerroum, 2020; Chandra, 2021; Kumar, Mahato, Kamle, Mohanta, & Kang, 2017). Aflatoxins pose significant health risks, including hepatotoxicity and carcinogenicity, with a particular impact on liver function (Dhakal, Hashmi, & Sbar, 2020; Rushing & Selim, 2019). Exposure to aflatoxins through contaminated food sources such as nuts, grains, and seeds can lead to both acute and chronic health issues. In the liver, aflatoxins are metabolized into reactive intermediates that cause cellular damage and mutations, thereby contributing to cancer development (Dhakal et al., 2020). It is important to note that aflatoxins refer to the toxic compounds produced by specific *Aspergillus* species, rather than the fungi themselves.

Metabolomics has emerged as a powerful tool for analyzing dynamic changes in metabolites and understanding plant-pathogen interactions. Through high-throughput technologies such as gas chromatography–mass spectrometry (GC–MS) and liquid chromatography-mass spectrometry (LC-MS), metabolomics enables the identification and quantification of metabolites involved in plant stress responses (Chen & Singer, 2007). For instance, Rubert et al. used metabolomics to detect mycotoxins and identify *Fusarium* species in wheat (Rubert et al., 2017), while Wang et al. identified resistance-related metabolites in Chinese poplar (*Hyphantria cunea*) using gas chromatography - time-of-flight mass spectrometry (GC-TOF/MS). Isoflavones, a class of plant secondary metabolites, play a significant role in plant defense mechanisms (Wang et al., 2017). Bednarek et al. noted that plants often increase the production of inherent defense compounds or synthesize new metabolites in response to disease or damage (Bednarek & Osbourn, 2009). Soybean isoflavones, such as daidzein and genistin, contribute to insect resistance, with increased levels of these compounds correlating with reduced pest damage (Zavala, Mazza, Dillon, Chludil, & Ballare, 2015). Murakami et al. also observed that damage from *Spodoptera litura* induces de novo synthesis of isoflavones, highlighting their role in the defense mechanisms of the plant (Murakami et al., 2014). Moreover, isoflavones are well-known for their diverse biological activities, including antibacterial properties. Studies have shown that isoflavones like genistein inhibit bacterial DNA replication, protein synthesis, and respiration by disrupting bacterial cell structures (Liu et al., 2016). Additionally, genistein inhibition of topoisomerase II has been linked to its antibacterial activity, while structurally similar compounds like daidzein show weaker activity (Verdrengh, Collins, Bergin, & Tarkowski, 2004). *Medicago truncatula* and *Lotus japonicus* produce specific isoflavonoids, such as medicarpin and vestitol, respectively, which are instrumental in combating pathogens like *Erysiphe pisi* (powdery mildew) and *Rhizoctonia solani* (Canivenc-Lavier & Bennetau-Pelissero, 2023; Yang & Wang, 2024). In *Phaseolus vulgaris* (common bean), isoflavones including genistein and its derivatives increase in concentration during infection by *Xanthomonas axonopodis*, indicating a direct link between isoflavone biosynthesis and resistance to bacterial blight (Cox et al., 2021). Medicarpin has been identified as a key compound providing resistance against *Erysiphe pisi*, the pea powdery mildew pathogen, in *Medicago truncatula* (Gupta et al., 2022). Additionally, rice lines expressing the soybean isoflavone synthase gene (*GmIFS1*) have been developed to enhance resistance against *Magnaporthe oryzae*, the causal agent of rice blast disease (Pokhrel, Ponniah, Jia, Yu, & Manoharan, 2021). Fava beans (*Vicia faba*) also synthesize isoflavones that contribute to their defense mechanisms against fungal pathogens, aiding in resistance to diseases such as powdery mildew (Wang, Li, & Luo, 2024). Furthermore, alfalfa and red clover utilize isoflavones as part of their defense strategies against various pathogens, with the accumulation of these compounds under stress conditions acting as antimicrobial and antifungal agents (Wang et al., 2024).

Given the significant economic losses caused by soybean field mold, developing mildew-resistant soybean varieties is a critical strategy for mitigating these effects. The limited availability of diverse disease-resistant soybean lines presents significant challenges, especially in regions with specific environmental conditions. This restricted genetic diversity impedes breeders ability to effectively introgress new resistance genes into high-yielding cultivars. Consequently, without a broad genetic base, breeding programs may struggle to develop soybean varieties that are both high-yielding and disease-resistant (Sang et al., 2023). Breeding mildew-resistant soybean varieties in areas characterized by high rainfall and humidity involves several specific obstacles. Elevated humidity and precipitation create favorable conditions for the proliferation of pathogens such as *Phytophthora* and powdery mildew, thereby increasing disease pressure and incidence. This necessitates the development of varieties capable of withstanding higher levels of pathogen exposure, complicating breeding efforts as resistance must remain robust under these challenging conditions (Ramalingam et al., 2020; Sang et al., 2023). Unlike single-gene resistance traits, such as those conferring resistance to *Phytophthora*, many forms of mildew resistance are quantitative and controlled by multiple genes. This polygenic nature complicates the identification and selection of resistant traits using traditional breeding methods. The complexity of these genetic interactions requires advanced breeding techniques, such as marker-assisted selection (MAS). However, MAS may not always yield clear results due to the intricate polygenic architecture of resistance. Additionally, breeding for mildew resistance can inadvertently affect other desirable agronomic traits, including yield and plant height. For instance, some breeding efforts aimed at enhancing white mold resistance have resulted in increased lodging due to changes in stem integrity, which negatively impacts overall yield. Balancing disease resistance with other agronomic traits remains a critical yet challenging aspect of soybean breeding (Dubey, Kumar, Abd_Allah, Hashem, & Khan, 2019). Conducting field trials in regions with high rainfall introduces variability in disease pressure across different years and locations, making it difficult to consistently evaluate the performance of resistant lines. This variability can lead to misleading results during the selection process, thereby complicating the identification of truly resistant genotypes (Sang et al., 2023). Furthermore, the genetic plasticity of pathogens such as *Phytophthora* poses an ongoing challenge, as these organisms can rapidly evolve in response to selective pressures from resistant cultivars. This adaptability means that even newly developed resistant varieties may become susceptible over time, necessitating continuous research and development efforts to maintain effective disease resistance (Hale, Brown, & Wijeratne, 2023; Kronmiller et al., 2023; Zhang et al., 2019).

While the antibacterial functions of flavonoids are well-documented, limited research has focused on the antifungal efficacy of soybean isoflavones against field mold. The synergistic presence of multiple isoflavones enhances antifungal activity through various mechanisms, primarily driven by their structural features and interactive effects. Isoflavones, a subclass of flavonoids predominantly found in legumes such as soybeans, exhibit specific structural characteristics that underpin their biological activities, including antifungal properties. These compounds contain hydroxyl groups (-OH) that are essential for their antifungal efficacy. The hydroxyl groups increase the solubility and reactivity of isoflavones, facilitating effective interactions with fungal

cell membranes and walls. The capacity to form hydrogen bonds with these structures disrupts cellular integrity, resulting in increased permeability and ultimately cell death (Křížová, Dadáková, Kašparovská, & Kašparovský, 2019; Rípodas, Dalla Via, Aguilar, Zanetti, & Blanco, 2013; Sohn et al., 2021). Isoflavones are present in both glycoside forms (e.g., genistin, daidzin) and aglycone forms (e.g., genistein, daidzein). Glycosylation often enhances the bioavailability and stability of isoflavones, thereby improving their antifungal efficacy. For example, studies have demonstrated that glycosides such as genistin and daidzin exhibit lower minimum inhibitory concentrations (MICs) against fungi compared to their aglycone counterparts (Davidova, Galabov, & Satchanska, 2024; Kisiriko et al., 2021; Silva-Beltran, Boon, Ijaz, McKinney, & Gerba, 2023). This enhanced antifungal activity of glycoside forms underscores the importance of considering both structural variants in the development of effective antifungal agents. Overall, the combination of multiple isoflavones, particularly in their glycoside forms, offers a promising strategy for enhancing antifungal activity through improved solubility, reactivity, and synergistic interactions with fungal pathogens.

Preliminary studies suggest that soybean isoflavones may exhibit significant antifungal activity, providing a foundation for developing mildew-resistant soybean varieties and natural inhibitors to control field mold. Understanding the antifungal mechanisms of soybean isoflavones is essential for future soybean breeding programs and microbial control strategies.

2. Materials and methods

2.1. Plant material and experimental design

The plant material for this study comprised nine summer-sown soybean varieties collected from the southwestern region of China. These varieties were categorized into four distinct groups based on their susceptibility to field mildew, following the mildew index of seeds (MIS) and mildew index of pods (MIP) as described in (Liu et al., 2016). The selected soybean varieties encompass a spectrum of mildew susceptibility, enabling a comprehensive assessment of their diverse genetic backgrounds in response to *Aspergillus flavus*. These varieties were chosen based on our previous studies (Deng JunCai et al., 2015; Liu et al., 2016). They were categorized into four distinct groups: Category I represents soybean pods and seeds that are highly susceptible to mildew (Mildew Susceptibility Index [MIS] $\geq$ 30; Mildew Infection Percentage [MIP] $\geq$ 50); Category II includes varieties where the seeds are highly susceptible to mildew (MIS $\geq$ 30) while the pods exhibit resistance (MIP $\leq$ 50); Category III comprises varieties that demonstrate strong resistance in both pods and seeds to mildew (MIS $\leq$ 10; MIP $\leq$ 50); and Category IV consists of varieties where the seeds are resistant to mildew (MIS $\leq$ 10) but the pods are susceptible (MIP $\geq$ 50). This classification provided a structured framework for assessing the mildew resistance of soybean seeds and pods under field conditions, enabling detailed investigation of their responses to mildew infection (Deng JunCai et al., 2015).

This experiment was divided into two parts. Experiment 1 involved a potted trial conducted in an artificial rainfall chamber (Deng JunCai et al., 2015). The experiment took place at the teaching and research park of Sichuan Agricultural University Ya'an campus. Soybean seeds were sown in mid-June, and two experimental groups were established: a mildew-induced treatment group and a control group under normal conditions. In this study, specific environmental parameters were meticulously monitored and controlled to establish optimal conditions for mildew development, while simultaneously maintaining control groups under non-inducing conditions. For example, control groups were likely maintained in environments with lower humidity levels or subjected to temperature extremes to inhibit fungal growth. Each treatment was repeated three times. Early management for both groups was identical to field experiments. Upon reaching the R8 (mature) stage, the treatment group was placed in the artificial rainfall chamber to simulate continuous rainy conditions, inducing mildew in the seeds, while the control group was kept dry in a drought shed. Some pods were harvested at the start of the experiment as controls (0 days). Seeds from the mildew-treated group were harvested seven days post-treatment (T), and those in the drought shed were harvested concurrently as control groups (CK). Control groups were maintained under standardized environmental conditions, excluding factors that could induce mildew. Key environmental variables such as humidity, temperature, and light exposure were carefully regulated to minimize their potential impact on the experimental outcomes. Proper management of these control conditions is essential to ensure that observed differences in plant responses are attributable to mildew induction rather than confounding environmental influences. All harvested seeds were stored at −80 °C for further analysis.

Experiment 2 was designed to verify the antibacterial activity of soybean isoflavones against *Aspergillus flavus*, a key pathogen responsible for field mildew in soybeans, which had been previously isolated and purified by from infected soybean pods (Deng JunCai et al., 2015; Liu et al., 2016). Six soybean isoflavone aglycones and glycosides were tested as antimicrobial agents, sourced from Weikeqi Bio-Technology Co., Ltd., China. The standards used were daidzein (DE, batch number: 130715), glycitein (GLE, batch number: 130716), genistein (GE, batch number: 130426), daidzin (DG, batch number: 131214), genistin (GEG, batch number: 130429), and glycitin (GLG, batch number: 130629). Fluconazole was used as the positive control for comparison in antimicrobial activity assays. Assays were conducted using serial dilution to assess the antifungal activity of isoflavones at multiple concentrations. A high-concentration working solution of the isoflavones was prepared, followed by serial dilution across the wells of a 96-well plate. This approach allows for testing a range of concentrations to determine the minimum inhibitory concentrations (MICs) of the isoflavones against *Aspergillus flavus*. The concentration range typically spanned from 200 $\mu g \cdot mL^{-1}$ (high concentration) to 0.1 $\mu g \cdot mL^{-1}$ (low concentration), depending on the dilution factor. The plates were incubated at 30 °C for 48 h, a condition optimal for fungal growth, allowing sufficient time for any antifungal effects to become evident. RPMI-1640 culture medium was used for both preparing the fungal suspension and diluting the isoflavones, as it supports fungal growth effectively. To ensure accurate results, the assays were performed in a sterile environment to minimize the risk of contamination, thereby confirming that observed effects were solely attributable to the isoflavones. The assay included seven replicates for each isoflavone concentration, providing a robust dataset to assess the variability and consistency of responses. A positive control (fluconazole) was placed in the eighth row of the 96-well plate to benchmark the isoflavones efficacy against a known antifungal agent. Additionally, growth control (without any isoflavones) and blank control (containing only the culture medium) were included to ensure accurate interpretation of the results.This methodology was adapted from our previous studies (Deng JunCai et al., 2015; Liu et al., 2016).

2.2. Equipment and materials

The analytical instruments used in this study included an agilent liquid chromatography-mass spectrometry (LC-MS) system, which consisted of the Agilent 1260 HPLC system and the Agilent 6120 single quadrupole mass spectrometer (Agilent Technologies, USA). The system was equipped with a Waters Xselect HSS T3 chromatographic column (2.1 mm × 100 mm, 2.5 μm). Other key equipment included a Milli-Q ultrapure water system (Millipore, USA), a Bullet Blender BB24-AU ball mill (Next Advance, USA), a freeze dryer (FDU-1110·2110, Eyela, Tokyo Rikakikai Co. Ltd), a constant temperature incubator, and a vortex oscillator (Scientific Industries, USA). Microscopic analysis was performed using an Olympus microscope (Japan), and sample measurements were taken using a Sartorius BSA224S-CW analytical balance (Germany) and Eppendorf pipettes (Germany). A blood cell counting

chamber (79 mm × 39 mm × 13 mm, Shanghai Qiujing) was used for cell quantification, and centrifugation was conducted with a BECKMAN Coulter Avanti J-25I centrifuge (USA). Assays were performed in 96-well flat-bottom microtiter plates (Corning, USA). Culture media used was RPMI 1640 (Fisher Scientific, USA). Organic phase needle filters (13 mm × 0.22 μm, Jinteng, Tianjin, China) and chromatographic-grade acetonitrile (Thermo Fisher Scientific) were used for sample preparation. All other reagents were of analytical grade and commercially available.

2.2.1. Isoflavone detection

The isoflavone content in soybean pods and seeds, both before and after mildew induction treatment, was determined using LC-MS (Wu et al., 2017).

2.2.2. Sample preparation

Approximately 50 mg of soybean pod and seed powder was accurately weighed and placed in a 10 mL centrifuge tube with a stopper. Then, 2.5 mL of 80 % methanol aqueous solution (solid-liquid ratio 1:40) was added. The tube was sealed and vortexed for 10 s, followed by ultrasonication in an ice-water bath (40 KHz, 300 W) for 3 h. After sonication, the mixture was centrifuged at 13,000 rpm for 10 min. A 1 mL aliquot of the supernatant was filtered through a 0.22 μm organic phase filter into a 1.5 mL vial for injection. The sample solution was stored at −20 °C until analysis by LC-MS. Each sample was prepared and analyzed five times.

2.2.3. Quantitative analysis

The mobile phase consisted of acetonitrile (A) and 0.1 % (*v*/v) acetic acid aqueous solution (B). The gradient elution program was: 0 to 9 min, 15 % to 20 % A; 9 to 18 min, 20 % to 40 % A; 18 to 21 min, 40 % A; 21.01 to 30 min, 15 % A. The flow rate was 0.3 mL/min, with the column maintained at 30 °C and an injection volume of 1 μL. The drying gas (N_2) flow rate was set at 10 L/min, with an atomizer pressure of 35 Psig and a drying gas temperature of 350 °C. The capillary voltage was 3800 V. Electrospray ionization (ESI) in positive ion mode was used, with selected ion monitoring (SIM) for quantitative analysis. The mass-to-charge ratios (*m/z*) used for target compounds were: 417 for daidzin (DG), 447 for glycitin (GLG), 433 for genistin (GEG), 503 for malonyldaidzin (MD), 533 for malonylglycitin (MGL), 459 for acetyldaidzin (AD), 519 for malonylgenistin (MG), 489 for acetylglycitin (AGL), 255 for daidzein (DE), 285 for glycitein (GLE), 475 for acetyldaidzein (AG), and 271 for genistein (GE).

2.3. Antibacterial activity assay

The antibacterial activity of six isoflavone aglycones and glycosides was assessed using the M38-P protocol recommended by the National Committee for Clinical Laboratory Standards (NCCLS) (Pierce, Uppuluri, Tummala, & Lopez-Ribot, 2010).

2.3.1. Preparation of bacterial suspension

The preserved *Aspergillus flavus* strains were transferred to potato dextrose agar (PDA) medium and cultured at 30 °C for 7 days. The spores were then transferred to a fresh medium and cultured for an additional 2 days to ensure good viability. To prepare the bacterial suspension, 1 mL of 0.85 % physiological saline was added to the surface of the culture, and the spores were gently collected with a pipette and transferred into a sterile test tube. Then, 5 mL of RPMI-1640 culture medium was added to dilute the spores. The mixture was allowed to stand for 3–5 min, and the upper suspension was collected and shaken on a vortex mixer for 15 s. The spore concentration was counted using a hemocytometer, and the suspension was diluted to a concentration of 1×10^6 spores/mL in RPMI-1640 culture medium.

2.3.2. Antibacterial testting protocol (inoculation)

RPMI-1640 medium was used to prepare the antibacterial agents into working solutions. Each drug was tested in seven replicates, with the eighth row used as a positive control (fluconazole). To begin, 200 μL of the high-concentration antibacterial working solution was added to the first column of a 96-well plate. Then, 100 μL of the culture medium was added to columns 2–11. Column 11 served as a growth control without the antibacterial agent, while column 12 served as a blank control, with only 200 μL of culture medium added. Serial dilutions were performed across columns 1–10 using a half-fold dilution method. Finally, 100 μL of the bacterial suspension (1×10^6 spores/mL) was added to columns 1–11. The plate was covered, sealed with sealing film, and incubated at 30 °C for 48 h. The end point was determined by visual inspection for fungal growth.

2.4. Results analysis

The MIC was defined as the lowest concentration of the antibacterial agent that completely inhibited visible fungal growth, determined by comparing the wells with test agents to the growth control well that contained no antibacterial agent. The results were evaluated following the M-27 A scoring standard, ensuring consistency in the determination of the MIC. This measure is crucial for evaluating the effectiveness of antimicrobial agents and assessing the susceptibility of microorganisms to specific drugs.

2.5. Statistical analysis and data visualization

Microsoft Excel was used for data organization, preliminary calculations, and preparation of data for further analysis. Raw data were processed and organized using the Agilent OpenLab workstation. Multivariate statistical analysis was conducted using SIMCA-P 13.0 software, which allowed for pattern recognition and identification of key metabolites. SPSS 23.0 was employed for variance analysis (ANOVA) to assess the significance of differences between experimental groups. Data visualization, including graphs and charts, was generated using both Excel and SIMCA-P 13.0 to effectively communicate the results.

3. Results

3.1. Isoflavones content changes in soybean seeds after mildew treatment

Based on previous research findings and the observed mildew resistance characteristics of soybean pods and seeds, the nine soybean varieties tested were categorized into four distinct groups (Table 1). Isoflavone content analysis in the seeds of these nine varieties revealed significant changes before and after mildew induction treatment compared to the control group. In general, the total isoflavone content in most varieties showed a decreasing trend post-treatment. However, in the fourth category, variety C103 exhibited a significant increase in total

Table 1
Information of soybean varieties with different mildew index.

Type	Variety Code	Seed mildew index (MIS)	Pod mildew index (MIP)	Seed coat color
I	GX	41.75	65.67	Yellow
	ND12	32.90	57.52	Yellow
	E70	53.37	84.57	Yellow
II	A13	53.07	47.1	Yellow
	D15	58.79	31.98	Yellow
	D1141	51.13	42.21	Yellow
III	D49	12.04	5.85	Brown
IV	2162	2.50	78.25	Black
	C103	1.61	70.19	Black

isoflavone content after treatment, with notable changes in the malonyl isoflavone glycoside levels. C103 also had the highest isoflavone content in seeds across all tested varieties. Among the different isoflavone types, the content of isoflavone aglycones showed the most pronounced changes across all varieties (Table 2). In most cases, the isoflavone aglycone content increased significantly after treatment, with the exception of varieties 2162 and D15, which exhibited a decrease in aglycone content following seven days of mildew induction treatment compared to the control group.

The isoflavone content in soybean seed pods before and after mildew induction treatment was analyzed (Table 3). The overall trend in total isoflavone content in the seed pods was consistent with the changes observed in the seeds, showing a general decrease across all nine soybean varieties. However, the pattern of change for isoflavone aglycones in the seed pods contrasted with that in the seeds, displaying a significant downward trend post-treatment. The other two types of isoflavones, malonyl isoflavones and glycosides, also exhibited a marked decrease after treatment. Notably, malonyl isoflavones were undetectable in all varieties except C103 following mildew induction. Additionally, acetylated isoflavone glycosides were not detected in any of the seed pod samples.

Table 2
The multiple comparison of isoflavones contents in seeds among different treatments.

Sample	TG (mg/g)	TM (mg/g)	TE (mg/g)	TT (mg/g)
ND12–0	1.0395 ± 0.0479a	1.6744 ± 0.0347a	0.0032 ± 0.0001b	2.7171 ± 0.0517a
ND12-CK	1.0450 ± 0.1168a	1.4924 ± 0.1635b	0.0027 ± 0.0002b	2.5401 ± 0.2790a
ND12-T	0.8431 ± 0.0351b	1.1145 ± 0.0337c	0.0239 ± 0.0036a	1.9815 ± 0.0658b
E70–0	0.7801 ± 0.2177a	1.5166 ± 0.5683a	0.0198 ± 0.0145a	2.3165 ± 0.7995a
E70-CK	0.7762 ± 0.1637a	1.4533 ± 0.3181a	0.0039 ± 0.0012b	2.2334 ± 0.4829a
E70-T	0.7987 ± 0.0678a	1.3006 ± 0.1223a	0.0307 ± 0.0027a	2.1300 ± 0.1882a
GX-0	0.4972 ± 0.0089b	1.0154 ± 0.0379a	0.0029 ± 0.0001b	1.5155 ± 0.0458b
GX-CK	0.6264 ± 0.1652a	0.9771 ± 0.1516a	0.0044 ± 0.0005b	1.6679 ± 0.0169a
GX-T	0.5559 ± 0.0121ab	1.0810 ± 0.0244a	0.0166 ± 0.0022a	1.6535 ± 0.0336a
A13–0	0.5428 ± 0.0456a	0.5999 ± 0.0771b	0 ± 0c	1.1427 ± 0.1227b
A13-CK	0.6300 ± 0.0166b	0.7027 ± 0.0145a	0.0112 ± 0.0017b	1.3439 ± 0.0324a
A13-T	0.6576 ± 0.0141b	0.6555 ± 0.0158ab	0.0194 ± 0.0008a	1.3325 ± 0.0287a
D15–0	0.5883 ± 0.0383a	1.0067 ± 0.0675a	0.0102 ± 0.0023a	1.6052 ± 0.1072a
D15-CK	0.5567 ± 0.0478a	0.9385 ± 0.0959a	0.0016 ± 0.0004b	1.4968 ± 0.1435a
D15-T	0.4557 ± 0.0249b	0.6367 ± 0.0795b	0.0021 ± 0.0021b	1.0944 ± 0.1062b
D1141–0	0.6128 ± 0.0343a	0.8102 ± 0.0553c	0.0036 ± 0.0001b	1.4266 ± 0.0890b
D1141-CK	0.5443 ± 0.0965a	0.9522 ± 0.0986b	0.0055 ± 0.0010b	1.5021 ± 0.0662b
D1141-T	0.5198 ± 0.0091a	1.2872 ± 0.0764a	0.0438 ± 0.0038a	1.8508 ± 0.0854a
D49–0	0.7142 ± 0.0611a	1.1223 ± 0.0980a	0.0067 ± 0.0006b	1.8432 ± 0.1591a
D49-CK	0.6033 ± 0.0353b	0.7710 ± 0.0782b	0.0034 ± 0.0002c	1.3777 ± 0.1133b
D49-T	0.5300 ± 0.0027c	0.5831 ± 0.0121c	0.0106 ± 0.0009a	1.1237 ± 0.0132c
C103–0	2.0349 ± 0.0479a	2.9317 ± 0.0347b	0.0469 ± 0.0001a	5.0135 ± 0.0517a
C103-CK	1.7476 ± 0.0412ab	2.6637 ± 0.0345b	0.0068 ± 0.0008c	4.4182 ± 0.0507b
C103-T	1.1379 ± 0.0141b	4.5441 ± 0.0870a	0.0138 ± 0.0013b	5.6958 ± 0.0956a
2162–0	1.4278 ± 0.0903a	2.2016 ± 0.1087a	0.0058 ± 0.0006c	3.6352 ± 0.1976a
2162-CK	1.4008 ± 0.0622a	2.0231 ± 0.0987b	0.0104 ± 0.0005a	3.4342 ± 0.1429a
2162-T	0.8578 ± 0.0165b	1.0105 ± 0.0334c	0.0072 ± 0.0003b	1.8754 ± 0.0496b

Note:TG, TM, TE, TT represent the content of β-Glucoside, Malonylglucoside, Aglycone, and total isoflavones. The data indicates mean ± SD. The same soybean germplasms data in one column with different small letter mean significant different at ($p \leq 0.05$) level. Post-hoc test was performed after ANOVA to identify which specific groups differed from each other.

Table 3
The multiple comparison of isoflavones contents in soybean pods among different treatments.

Sample	TG (mg/g)	TM (mg/g)	TE (mg/g)	TT (mg/g)
ND12–0	0.4499 ± 0.0028a	0.4798 ± 0.0140a	0.0060 ± 0.0003a	0.9357 ± 0.0162a
ND12-CK	0.4117 ± 0.0029b	0.2078 ± 0.0014b	0.0030 ± 0.0002b	0.6225 ± 0.0044b
ND12-T	0.4171 ± 0.0056b	0 ± 0c	0.0029 ± 0.0002b	0.4200 ± 0.0056c
E70–0	0.4460 ± 0.0039b	0.3402 ± 0.0019a	0.0085 ± 0.0008b	0.7947 ± 0.0057b
E70-CK	0.4545 ± 0.0030a	0.3407 ± 0.0022a	0.0117 ± 0.0002a	0.8069 ± 0.0046a
E70-T	0.4098 ± 0.0057c	0 ± 0b	0.0054 ± 0.0004c	0.4153 ± 0.0055c
GX-0	0.4576 ± 0.0007a	0.3405 ± 0.0025a	0.0076 ± 0.0012a	0.8057 ± 0.0041a
GX-CK	0.4367 ± 0.0068b	0.3291 ± 0.0029b	0.0077 ± 0.0009a	0.7735 ± 0.0102b
GX-T	0.4095 ± 0.0043c	0 ± 0c	0.0059 ± 0.0002b	0.4154 ± 0.0044c
A13–0	0.4115 ± 0.0062a	0.0008 ± 0.0003a	0.1585 ± 0.0052b	0.5708 ± 0.0102b
A13-CK	0.4118 ± 0.0023a	0.0004 ± 0.0002b	0.2893 ± 0.0067a	0.7015 ± 0.0073a
A13-T	0 ± 0b	0 ± 0c	0.1324 ± 0.0091c	0.1324 ± 0.0091c
D15–0	0.4575 ± 0.0028b	0.2068 ± 0.0007b	0.0048 ± 0.0002b	0.6691 ± 0.0032b
D15-CK	0.6240 ± 0.0150a	0.2374 ± 0.0079a	0.0179 ± 0.0063a	0.8793 ± 0.0147a
D15-T	0.4129 ± 0.0046c	0 ± 0c	0.0134 ± 0.0004a	0.4263 ± 0.0043c
D1141–0	0.5294 ± 0.0034a	0.3859 ± 0.0029a	0.0101 ± 0.0002b	0.9254 ± 0.0063a
D1141-CK	0.5132 ± 0.0019b	0.3644 ± 0.0022b	0.0177 ± 0.0041a	0.8953 ± 0.0057b
D1141-T	0.4111 ± 0.0047c	0 ± 0c	0.0108 ± 0.0006b	0.4220 ± 0.0046c
D49–0	0.4844 ± 0.0050a	0.3604 ± 0.0038a	0.0091 ± 0.0002a	0.8539 ± 0.0084a
D49-CK	0.4699 ± 0.0052b	0.3517 ± 0.0055b	0.0081 ± 0.0005b	0.8297 ± 0.0105b
D49-T	0 ± 0c	0 ± 0c	0.0076 ± 0.0004b	0.0076 ± 0.0004c
C103–0	0.4432 ± 0.0042b	0.3879 ± 0.0061a	0.0107 ± 0.0006b	0.8419 ± 0.0098a
C103-CK	0.4493 ± 0.0047a	0.3752 ± 0.0133b	0.0254 ± 0.0032a	0.8499 ± 0.0208a
C103-T	0.4117 ± 0.0019c	0.2030 ± 0.0010c	0.0063 ± 0.0004c	0.6210 ± 0.0032b
2162–0	0.4455 ± 0.0050a	0.3630 ± 0.0043a	0.0062 ± 0.0008a	0.8147 ± 0.0097a
2162-CK	0.4400 ± 0.0051a	0.3595 ± 0.0034a	0.0065 ± 0.0006a	0.8059 ± 0.0083a
2162-T	0.4138 ± 0.0023b	0 ± 0b	0.0057 ± 0.0003a	0.4195 ± 0.0024b

Note:TG, TM, TE, TT represent the content of β-Glucoside, Malonylglucoside, Aglycone、and total isoflavones. The data indicates mean ± SD. The same soybean germplasms data in one column with different small letter mean significant different at ($p \leq 0.05$) level. Post-hoc test was performed after ANOVA to identify which specific groups differed from each other.

3.2. Metabolomic analysis of isoflavones in moldy soybeans

3.2.1. Isoflavone metabolic characteristics in soybean pods and seeds before and after mildew treatment

Based on the susceptibility of seeds to mildew, the nine soybean varieties were divided into two categories: Category I, which included soybean seeds susceptible to mildew (ND12, A13, E70, D1141, D15, GX), and Category II, which included soybean seeds resistant to mildew (C103, 2162, D49). The results of discriminant analysis showed significant changes in isoflavone metabolism after mildew treatment in the mildew-susceptible seeds(Fig. 1-A). The compounds contributing most to this metabolic variation were DE, GE, GLE, and T-E(Fig. 1-a). In contrast, the mildew-resistant seeds (Category II) exhibited minimal metabolic changes in response to mildew induction. Similarly, soybean seed pods were categorized according to their resistance to mildew: Category I for mildew-susceptible seed pods (ND12, E70, GX, C103, 2162) and Category II for mildew-resistant seed pods (D1141, D15, A13, D49). Interestingly, the metabolic response in seed pods was opposite to that observed in seeds. Pod-susceptible materials exhibited minimal changes in isoflavone metabolism after mildew treatment, while significant variation was detected in the mildew-resistant pod materials (Fig. 1-B). The GLE was identified as the compound contributing most to the discriminant model in the pods (Fig. 1-b).

3.2.2. Differential variable analysis of mildew-induced isoflavone metabolism

The differential variables related to mildew resistance in soybean varieties were identified by integrating isoflavone data obtained before and after mildew treatment with the grain and seed pod mildew index, followed by OSC-PLS-DA analysis. The soybean varieties were divided into four categories according to their mildew resistance (Table 1). Model evaluation parameters, including R2X, R2Y, and Q2, were derived from seven-fold cross-validation (Table 4). R2X reflects the

Table 4
Model evaluation parameters of seven times cross validation.

n	R^2X(cum)	R^2Y(cum)	Q^2(cum)
A	0.990	0.795	0.747
B	1.000	0.779	0.706
C	0.997	0.710	0.664
D	0.993	0.948	0.943

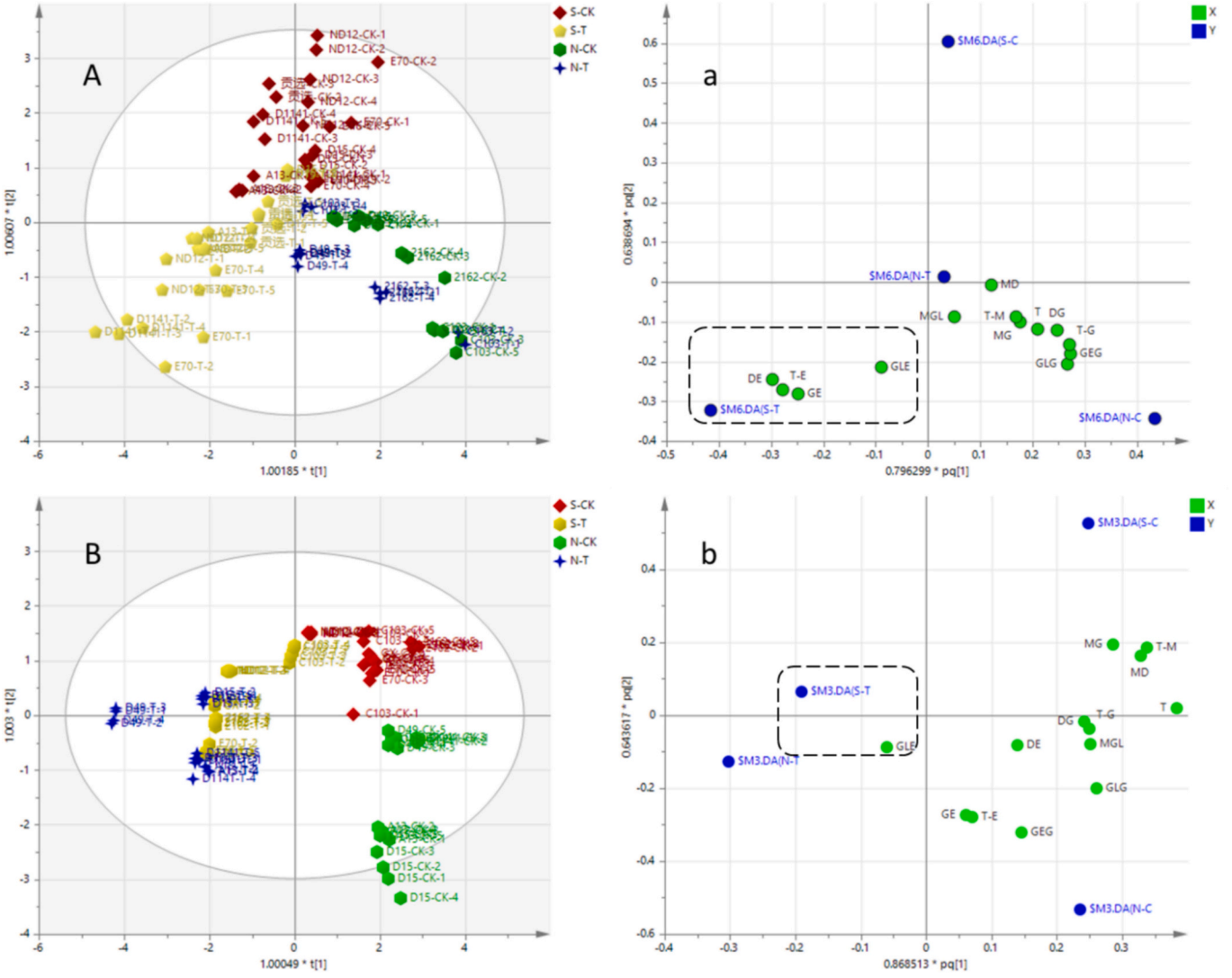

Fig. 1. Effect of mildew induction treatment on soybean metabolome. A and a are the score charts and load charts before and after grain mildew, respectively; B and b are the score charts and load charts before and after pod mildew, respectively; S-CK represents the control group of susceptible material, and S-T represents the susceptible material. Mildew induction group; N-CK represents the anti-mildew material control group, N-T represents the anti-mildew material mildew induction group.

cumulative explanation rate of the X matrix, while R2Y denotes the model's explanatory capability, and Q2 measures the prediction ability. The closer these values are to 1, the more stable and reliable the model. As seen in Table 4, R2X values for all models were above 0.9, indicating excellent model stability and reliability. Q2 values exceeding 0.9 after seed pod mildew treatment suggest strong predictive power in distinguishing post-treatment samples. However, the remaining models displayed weaker discriminative and predictive capacities.

The score chart for the control group (Fig. 2-A) clearly distinguishes between the two varieties, C103 and 2162, both of which have grains that are not susceptible to mildew. This classification is further supported by the load chart (Fig. 2-B), where the blue dots represent the corresponding groups from Fig. 2-A. The proximity of each principal component to these points reflects its contribution to the model's differentiation. The compounds that contribute most to the model's ability to distinguish between these groups are GEG, TG, MG, TT, T-M, and DG, as detailed in Table S1.

In the treatment group score chart (Fig. 2-B), a clear distinction between the groups is also observed, particularly in varieties C103, 2162, and D49, which are resistant to mildew. The load chart (Fig. 2-b) indicates that the compounds contributing to the discrimination in this model are GLG, MG, TT, TM, GEG, TG, MD, and DG. From the combined results of these two discriminant analyses, it is evident that the content of inherent components such as GLG, MG, TT, T-M, GEG, T-G, MD, and DG is higher in varieties whose seeds are resistant to mildew. After mildew induction treatment, the levels of GLG, TM, MD, TT, TG, and DG in the seeds remained relatively high. Comparative analysis suggests that GLG and MD are typical differential metabolites after soybean mildew induction treatment and could be further explored as candidate compounds with potential antibacterial properties (Fig. 2, Tables 2, 3).

The score chart of the control group (Fig. 3-C) shows clear differentiation between D1141, A13, and D15, which are varieties with seed pods resistant to mildew. The load chart (Fig. 3-c) highlights the compounds that contribute to this classification: GEG, GE, TE, TG, DG, GLG, DE, and MGL. Notably, the content of GLE in the seed pods of D49, a mildew-resistant variety, is particularly high. In the treatment group score chart (Fig. 3-D), there is also a clear distinction between the groups, with C103, 2162, and D49 being well differentiated. The load chart (Fig. 3-d) reveals that the key compounds contributing to this model are DE, T-E, G-E, and GLE. Based on these two sets of

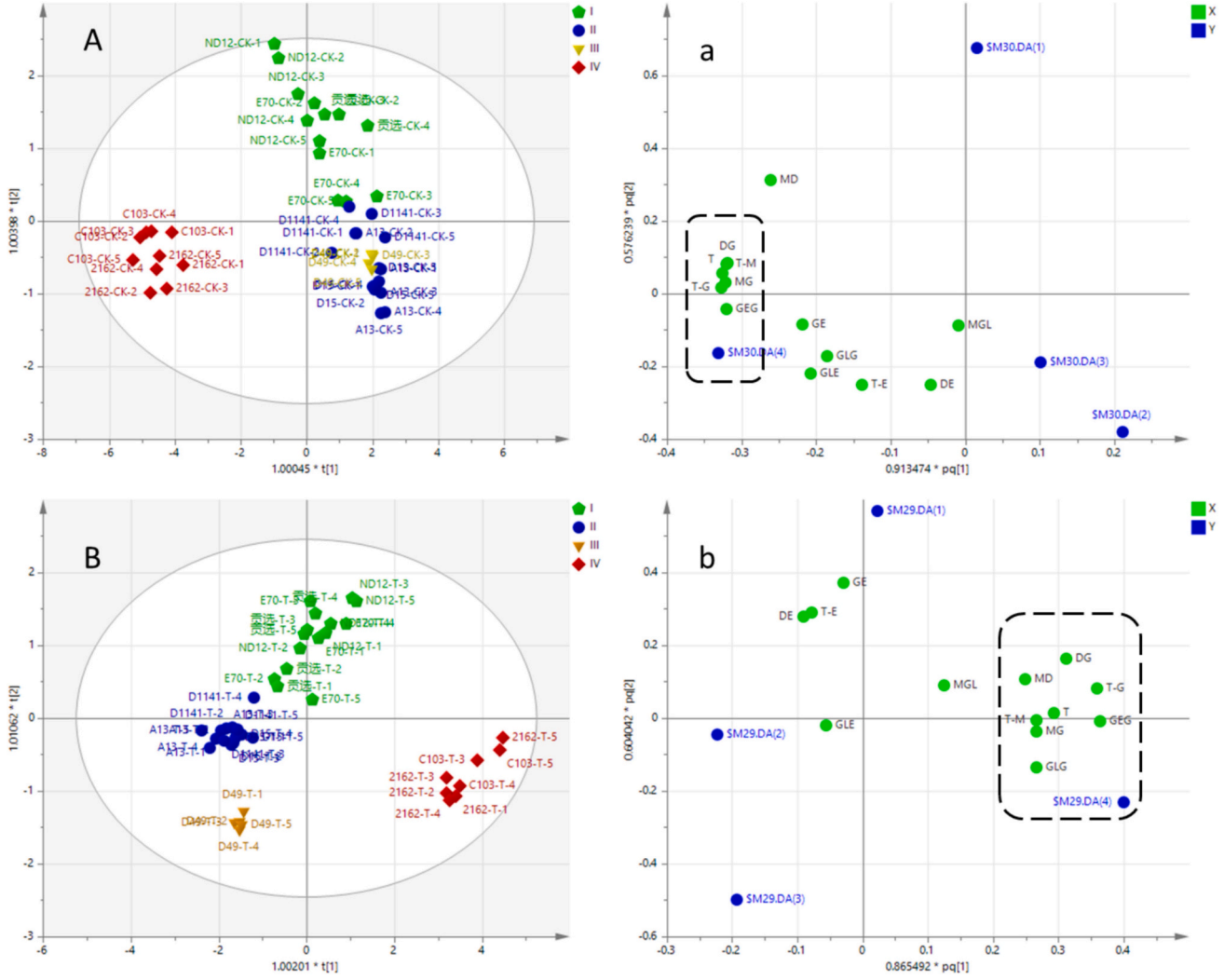

Fig. 2. Difference analysis before and after induction of mildew in grains of different materials (OSC-PLS-DA). A and a are the score map and load map of the soybean seeds and control group respectively, and B and b are the score map and load map of the seeds mildew treatment respectively. Fig. A: I represents GX, ND12, E70 seeds control group samples (CK), II represents A13, D15, D1141 seeds control group samples (CK), III represents D49 seeds control group samples (CK), IV represents 2162 and C103 seeds control group samples (CK). Fig. B: I represents GX, ND12, E70 seeds mildew treatment group samples (T), II represents A13, D15, D1141 seeds mildew treatment group samples (T), III represents D49 seeds mildew treatment group samples (T), IV represents 2162 and C103 seeds mildew treatment group samples (T).

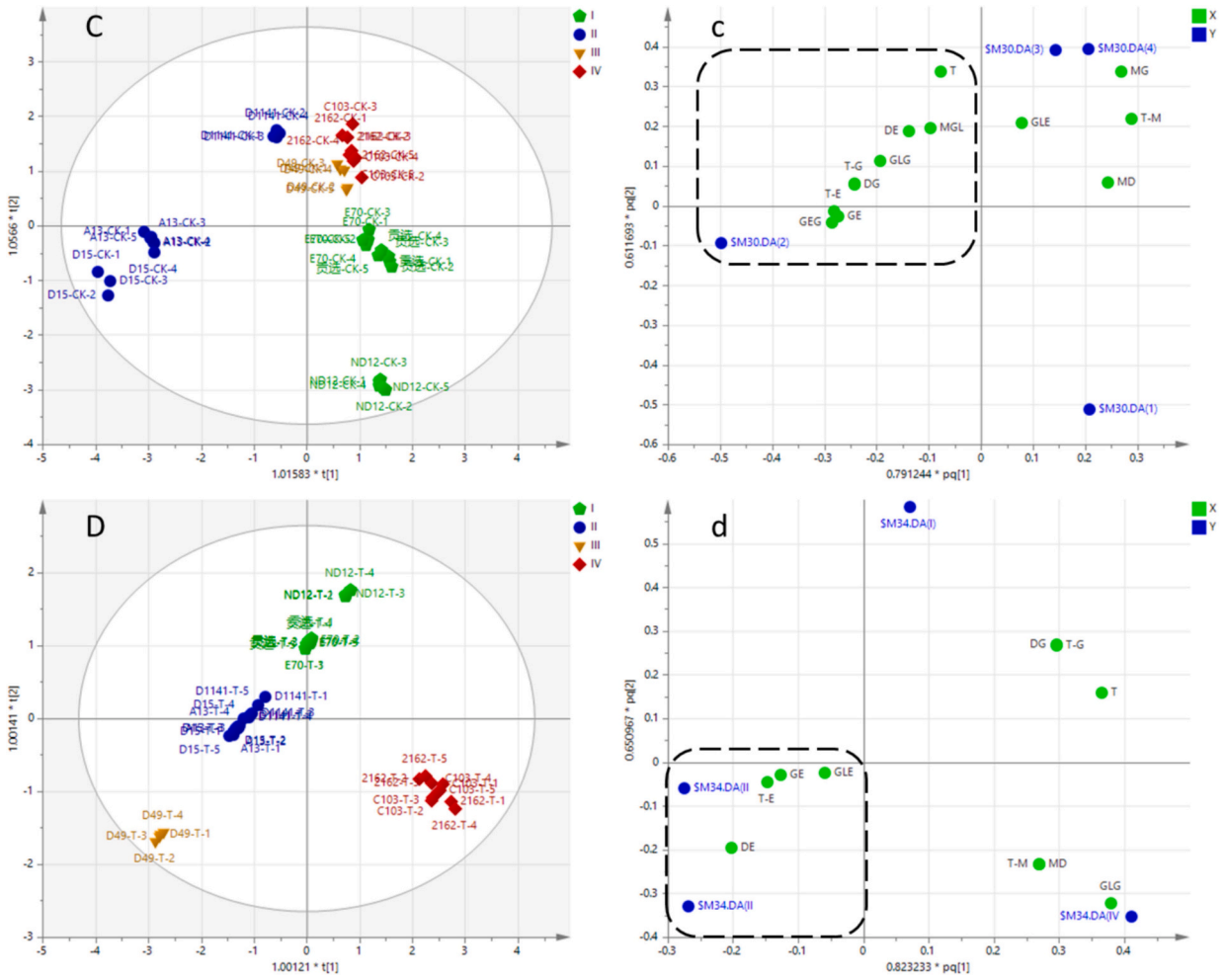

Fig. 3. Difference analysis of seed pods of different materials before and after mildew induction (OSC-PLS-DA). C and c are the score map and load map of the grain control group respectively. D and d are respectively the score chart and load chart after grain mildew treatment. Fig. C: I represents GX, ND12, E70 seed pods control group samples (CK), II represents A13, D15, D1141 seed pods control group samples (CK), III represents D49 seed pods control group samples (CK), IV represents 2162 and C103 seed pods control group samples (CK). Fig. D: I represents GX, ND12, E70 seed pods mildew treatment group samples (T), II represents A13, D15, D1141 seed pods mildew treatment group samples (T), III represents D49 seed pods mildew treatment group samples (T), IV represents 2162 and C103 seed pods mildew treatment group samples (T).

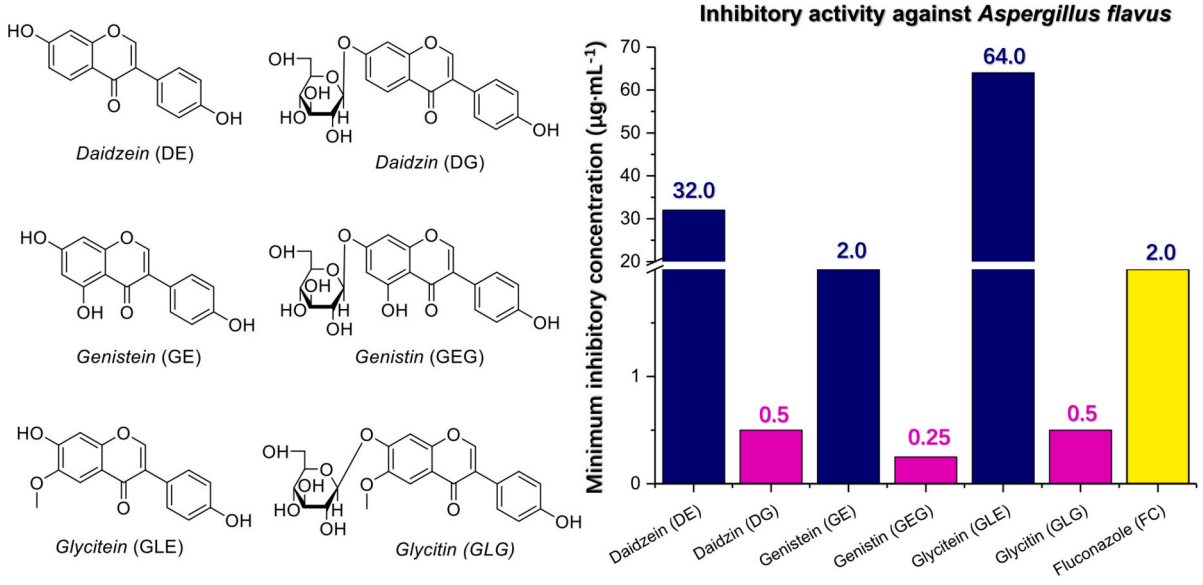

Fig. 4. Structures of soy isoflavones and their inhibitory activity against *Aspergillus flavus*.

metabolomic discriminant analyses, it is evident that after mildew induction, the isoflavone content in mildew-resistant seed pods shows a significant decreasing trend. The resistance activity in these varieties appears to rely on the consumption of inherent isoflavones. Specifically, GEG, T-G, DG, GLG, and MGL are identified as differential metabolites, which may serve as candidate compounds for further exploration of their antibacterial properties.

3.3. Antibacterial activity of isoflavones against aspergillus flavus: Glycosides exhibit superior activity compared to aglycones

The antibacterial activity of six isoflavones against *Aspergillus flavus* was evaluated by determining the minimum inhibitory concentrations (MICs) for each compound, as shown in Fig. 4. The results revealed that genistin, a glycoside, exhibited the lowest MIC of 0.25 $\mu g \cdot mL^{-1}$, followed closely by daidzin at 0.5 $\mu g \cdot mL^{-1}$. These values indicate that both glycosides possess strong antibacterial activity, comparable to the antifungal agent fluconazole, which had an MIC of 2.0 $\mu g \cdot mL^{-1}$ (Fig. 4). In contrast, the aglycones glycitein and daidzein demonstrated higher MICs of 64.0 $\mu g \cdot mL^{-1}$ and 32.0 $\mu g \cdot mL^{-1}$, respectively, suggesting reduced efficacy against *Aspergillus flavus*. Notably, while isoflavone aglycone and β-glucoside exhibited potent inhibitory effects, the activity of aglycone was significantly lower, emphasizing the superior antibacterial properties of isoflavone glycosides over their aglycone counterparts (Fig. 4). The observed antibacterial activity trend among the tested isoflavones can be summarized as follows: G-type (genistein) > D-type (daidzein) > GL-type (glycitein). In general, glycosides exhibited stronger antibacterial effects against *Aspergillus flavus* compared to their aglycone forms (Fig. 4).

4. Discussion and conclusion

Isoflavones, a class of naturally occurring compounds, are predominantly found in the legume family (*Fabaceae*), with soybeans being the primary dietary source for humans and a significant component of livestock feed (Polturak et al., 2023). Globally, soybean represent the fourth largest crop after major cereals such as maize, wheat, and rice, which constitute the primary staple foods for human consumption. Given their rich isoflavone content, soybean and soy-derived products are integral to human nutrition (Miladinović et al., 2019). Isoflavones have been associated with various health benefits, with preclinical studies providing strong evidence for their protective roles in preventing cardiovascular diseases, breast and prostate cancers, and alleviating postmenopausal symptoms (Martin, 2018). However, soybean production faces challenges due to environmental factors, particularly in regions like southwest China, where continuous rainfall during the summer harvest season often results in significant crop losses. The persistent humidity during this period hampers the timely harvesting of mature soybeans and creates conditions conducive to the proliferation of field fungi. These fungi infest the soybean plants, leading to FM, which is characterized by pre-harvest seed deterioration. This mold not only reduces seed quality but also has a detrimental impact on overall soybean yield (Liu et al., 2017). Therefore, addressing the effects of environmental stressors such as prolonged rainfall and FM is crucial for ensuring sustainable soybean production.

Mildew in soybean seed pods has been shown to alleviate mildew in seeds to a certain extent. Research has demonstrated significant differences in the content of specific isoflavones, such as GL-type isoflavones and isoflavone aglycones, in soybean seeds with varying mildew resistance, suggesting that these compounds may act as potential antibacterial agents. This aligns with previous findings that isoflavonoids function as phytoalexins, compounds produced by plants in response to stress or pathogen attacks (Li et al., 2021; Liu et al., 2017). The decrease in isoflavones in soybean seed pods following mildew treatment can be attributed to a combination of biological and environmental factors, which significantly influence the overall resistance of the soybean plant to mildew. Upon infection by *Aspergillus flavus*, soybean plants often redirect their metabolic resources towards the synthesis of other defensive compounds, rather than maintaining isoflavone levels (Khare et al., 2020; Nag, Lone, Praharaju, Khan, & Hussain, 2024; Tian, Lee, Woo, & Chun, 2020). This shift is part of the plant stress response, in which the production of phytoalexins, such as glyceollin, may be prioritized over isoflavones like malonylglucosides (Vidya et al., 2021). Consequently, the concentration of isoflavones, including malonylglucosides, may decrease substantially after mildew induction. Infection by pathogens can also trigger catabolic pathways that degrade existing isoflavones as part of the plant's defense mechanisms. During pathogen attacks, certain isoflavone compounds may be broken down to release energy or to form new defensive molecules(Zida, Bamba, Yacouba, Ouedraogo-Traore, & Guiguemdé, 2017; Zida, Néya, Soalla, Sérémé, & Lund, 2018). This degradation process contributes to the lower detectable levels of isoflavones in infected seed pods. Environmental conditions, such as humidity, temperature, and light exposure, also play a critical role in isoflavone biosynthesis and accumulation. High humidity, which is often associated with fungal infections, may create conditions less favorable for isoflavone synthesis (Deng JunCai et al., 2015). Moreover, light conditions can influence the expression of key enzymes involved in the biosynthetic pathway of isoflavones (Li et al., 2021). Isoflavones are known for their antifungal properties, and a decrease in their concentration could impair the pod's ability to resist mildew effectively. Compounds like genistin and daidzin have demonstrated significant antifungal activity against pathogens such as *Aspergillus flavus* (Liu et al., 2016). Reduced levels of these isoflavones may weaken the plant defense against subsequent infections, making the plant more susceptible to further fungal or bacterial attacks. Seed pods are vital for reproductive success, as they protect developing seeds and contribute to overall yield. If mildew infection leads to a decrease in isoflavone levels and, consequently, reduced pod resistance, it could result in higher rates of seed damage and lower seed viability (Zida et al., 2017). This would negatively impact soybean yield and quality. Furthermore, the reduction in isoflavones may increase the plant's susceptibility to other pathogens or pests, compounding the effects of mildew infection. As the plant defenses are compromised, it becomes more vulnerable to secondary infections, further impairing reproductive outcomes (Li et al., 2021). Overall, the decrease in isoflavones in soybean seed pods following mildew treatment can be attributed to metabolic reallocation, degradation processes, and environmental stress factors, which align with findings from previous studies (Deng JunCai et al., 2015; Li et al., 2021; Liu et al., 2016).

However, there is a lack of control and comprehensive investigation into their antibacterial activity against major pathogenic bacteria in the field. In this study, artificial mildew induction was applied to nine soybean materials with different mildew resistance characteristics. The isoflavone content in different parts of the soybean was monitored before and after mildew exposure. Metabolomic analysis identified G-type and M-type isoflavones as potential antibacterial metabolites, with an increase in isoflavone content observed in mildew-resistant grain materials post-mildew. This observation supports previous studies indicating that isoflavonoid levels in soybeans rapidly increase in response to pathogen treatment (Jeandet, Clément, Courot, & Cordelier, 2013; Lozovaya et al., 2004). The higher inherent isoflavone content in seed pod-resistant materials appears to play a role in mitigating mildew, consistent with earlier research showing that isoflavonoid biosynthesis helps resist biotic stress (Kim, 2022; Trush & Pal'ove-Balang, 2023). Isoflavones act as phytoalexins, antimicrobial compounds produced by plants in response to pathogen attack. These compounds play a crucial role in plant defense by inhibiting the growth and reproduction of fungi, thereby strengthening the plant's resistance to infections. Isoflavones such as genistein and daidzein disrupt fungal cell wall synthesis and function, leading to compromised integrity of the fungal membrane and, ultimately, cell lysis (Wang et al., 2024). In addition to their direct antifungal activity, isoflavones exhibit broad-spectrum antimicrobial

properties. Studies have demonstrated that they can inhibit the growth of various fungal pathogens, including *Fusarium graminearum*, *Sclerotinia sclerotiorum*, and *Colletotrichum truncatum*, by interfering with critical growth processes (Jiang, Haudenshield, & Hartman, 2012; Wang et al., 2024). Isoflavones may also enhance the effectiveness of other plant defense compounds through synergistic interactions, providing a more comprehensive and robust defense against fungal pathogens (Wang et al., 2024). This collaborative defense mechanism helps to ensure the plant's survival and resilience in the face of pathogen attacks.

The metabolomic analysis revealed that isoflavone aglycones are particularly sensitive to mildew stimulation. This is consistent with previous findings that rapid increases in isoflavonoid levels occur upon exposure to pathogens and that isoflavone aglycones are responsive to biotic stress (Jeandet et al., 2013; Lozovaya et al., 2004). Different parts of soybean varieties displayed distinct metabolic responses to mildew, corroborating earlier studies that also observed differential metabolic responses depending on the plant part (Liu et al., 2016). Further, antibacterial activity tests confirmed that G-type isoflavones, especially genistein, have strong antibacterial effects against *Aspergillus flavus*, in line with previous findings that genistein is particularly effective (Křížová et al., 2019). Isoflavones belong to the broader isoflavonoid family, and their biological activity is closely related to the number and positioning of hydroxyl groups on the molecule (Sohn et al., 2021). This relationship mirrors earlier research on flavonoids, which also emphasized the role of hydroxylation in enhancing biological activity (Li et al., 2023; Shamsudin et al., 2022). For example, the superior antibacterial activity of genistein may be attributed to its additional hydroxyl group, which enhances its efficacy against *Aspergillus flavus* and aflatoxins compared to other isoflavones. In contrast, isoflavone glycosides, which have a sugar group attached at position 7 of the A ring, generally exhibit reduced biological activity. This finding is consistent with previous studies suggesting that sugar substitution typically diminishes the activity of these compounds (Das et al., 2024; Jiang et al., 2010). In our study, genistein and genistin, among six isoflavones (both glycosides and aglycones) tested, displayed the strongest inhibitory effects against *Aspergillus flavus*. However, some studies have indicated that the introduction of sugar substituents does not necessarily increase inhibitory activity, although the overall effectiveness of the compounds may still be retained (Ryu et al., 2009; Xiao, 2017).

The identification of specific isoflavone metabolites in soybean varieties offers valuable markers for selective breeding or genetic modification aimed at enhancing mildew resistance. Through MAS, specific isoflavones—such as daidzein, genistein, and their glycosides (e.g., daidzin, genistin)—can be quantified in soybean seeds and pods. These metabolites have been associated with antifungal properties against pathogens like *Aspergillus flavus* (Deng JunCai et al., 2015; Liu et al., 2016; Wu et al., 2017). By measuring the levels of these isoflavones in different soybean varieties, breeders can select for those with higher concentrations of these compounds, which are linked to improved resistance to mildew. This approach allows for more efficient selection compared to traditional phenotypic assessments (Castro-Moretti, Gentzel, Mackey, & Alonso, 2020; Deng JunCai et al., 2015; Wu et al., 2017). Understanding the biosynthetic pathways of isoflavones can also facilitate genetic modifications aimed at increasing their production in soybean plants. For instance, overexpression or modification of genes involved in the synthesis of isoflavones could enhance their accumulation (Wang et al., 2024; Wu et al., 2017; Zhang, Zhang, Luo, Li, & Qin, 2022). Genetic engineering techniques could be employed to introduce or enhance genes responsible for the production of antifungal isoflavones in susceptible soybean varieties, potentially improving their resistance to mildew (Deng JunCai et al., 2015; Liu et al., 2016; Wang et al., 2024). Research findings that correlate higher levels of specific isoflavones with increased resistance to mildew can serve as a foundation for guiding breeding programs (Deng JunCai et al., 2015; Liu et al., 2016). If certain isoflavone profiles consistently align with resistance traits, these profiles can be used as reliable indicators during selection processes (Wu et al., 2017). Furthermore, conducting field trials that measure both isoflavone metabolite levels and the corresponding mildew resistance under natural conditions will help validate these markers and ensure their practical applicability in breeding programs (Deng JunCai et al., 2015; Liu et al., 2016; Wu et al., 2017). The precise mechanism underlying the enhanced effectiveness of isoflavone glycosides compared to aglycones in inhibiting *Aspergillus flavus* remains unclear and warrants further investigation. Additionally, the roles of G-type and M-type isoflavone glycosides, which were identified in this study, require further exploration to better understand how these metabolites contribute to the observed antifungal activity. Future research should focus on elucidating the specific mechanisms by which these metabolites exert their effects.

CRediT authorship contribution statement

Nishbah Mughal: Writing – review & editing, Writing – original draft, Validation, Formal analysis. **Xiaowen Zhang:** Visualization, Validation, Software, Investigation, Formal analysis, Data curation. **Noman Shoaib:** Writing – review & editing, Writing – original draft, Visualization, Validation. **Juncai Deng:** Visualization, Software, Formal analysis, Conceptualization. **Jinya Guo:** Visualization, Validation, Resources, Formal analysis. **Jing Zhang:** Writing – review & editing. **Wenyu Yang:** Visualization, Validation, Supervision, Resources, Investigation, Funding acquisition. **Jiang Liu:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Investigation, Funding acquisition, Data curation.

Declaration of competing interest

The authors affirm that they have no known financial or interpersonal conflicts that might have appeared to have impacted the research presented in this study.

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Author contributions

NM, and JL came up with the initial concept; XZ and JD did the experiments, NM and NS wrote the manuscript; created the figures, tables, and preliminary discussions; and JL, JG, WY and JZ, revised the manuscript. The manuscript has been approved by all authors.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102195.

Data availability

Data will be made available on request.

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