Changing light promotes isoflavone biosynthesis in soybean pods and enhances their resistance to mildew infection

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ABSTRACT

Mildew severely reduces soybean yield and quality, and pods are the first line of defense against pathogens. Maize-soybean intercropping (MSI) reduces mildew incidence on soybean pods; however, the reason remains unclear. Previous studies confirmed the key function of soy isoflavone in soybean mildew resistance, while changing light (CL) from maize shading is the most important environmental feature in MSI. CL also regulates isoflavone biosynthesis in soybean seeds. We hypothesized that CL affects isoflavone accumulation in soybean pods, impacting their disease resistance. In the present study, shading treatments were applied during different developmental stages of soybean plants according to various CL environments under MSI. Chlorophyll fluorescence imaging (CFI) and classical evaluation methods confirmed that CL, especially vegetative stage shading (VS), enhances pod resistance to mildew. Further metabolomic analyses and exogenous inhibitor experiments revealed the important relationship between jasmonic acid (JA) and isoflavone biosynthesis, which has a synergistic effect on the enhanced resistance of CL-treated pods to mildew. VS promoted the biosynthesis and accumulation of constitutive isoflavones upstream of the isoflavone pathway, such as aglycones and glycosides, in soybean pods. When mildew infects pods, endogenous JA signaling stimulates the biosynthesis of downstream inducible malonylated isoflavones and glyceolin to improve pod resistance.

KEYWORDS: intercropping; changing light; soybean pod; mildew; metabolomic; isoflavone

1 INTRODUCTION

Intercropping involves the growth of two or more crops on the same piece of land, and the period of symbiosis among different crops may be long or short. As an important agronomic strategy, intercropping has many benefits, including highly efficient utilization of resources, weeds, pests and disease control, and soil fertility improvement. Although intercropping has been used for several centuries in traditional agriculture, it is still common globally, especially in developing countries(Iqbal et al., 2019). The maize-soybean intercropping (MSI) system is a successful case that provides massive advantages in low-input and high-risk environments. As explained by biodiversity theory, maize-soybean intercropping systems are an ecological strategy to control or relieve diseases and insect pests; optimized field allocation in maize-soybean intercropping can reduce pests and diseases(Du et al., 2018).

As a serious plant disease, field mildew (FM), which is caused by the infection of *Fusarium verticillioides* during the wet season before soybean harvest, results in significant soybean yield losses(Liu et al., 2017). Our multiyear survey of disease dynamics confirmed that the FM rate of soybean plants growing under the MSI system was significantly lower than that of soybean plants growing alone (will be published elsewhere). There are multiple aspects underlying these findings. One important point is that soybean plant chemicals are affected by the intercropping environment, especially the light conditions, including light quality and intensity(Liu, Yang, et al., 2016). The soybean pod is the first line of defense for this leguminous seed against pests and pathogens, and pod chemicals play an important role(Deng et al., 2017). Our previous research showed that the chemicals in soybean pods can

mitigate the damage to the seed arising from FM at harvest time(Liu, Deng, et al., 2016).

In agricultural practice, MSI is employed in two ways: intercropping (IT) and relay intercropping (RIT); their marked difference is the shading periods from the maize(WANG Yi, 2016). In the IT system, maize and soybean are sown at the same time, and there is no shading from maize on the soybean plants in the vegetative stage. With the development of maize and soybean, when soybeans enter the reproductive stage, the lush leaves of maize result in shading of the soybean plant. Inversely in the RIT system, the main shading period is during the vegetative stage of the soybean; when soybean plants enter the reproductive stage, the maize is harvested, which increases light in the soybean canopy. These different symbiotic periods cause a changing light environment (CLE) for soybean plants in the IT and RIT systems: from bright to weak with IT and from weak to bright with RIT(F. Yang et al., 2014). Based on our initial research data and other publications, we hypothesized that a special CLE is one reason intercropped soybeans have higher FM resistance and that the CLE elevates the resistance of soybean pods via secondary metabolism. Many studies have shown that different light qualities or light intensities could affect the secondary metabolism of crops(Rechner, Neugart, Schreiner, Wu, & Poehling, 2016). However, the evidence and mechanisms revealed so far are limited. Although too many studies have focused on regulating crop secondary metabolism by uniform light, CLEs have rarely been studied.

In this current research, we conducted a field shade experiment to simulate the CLEs under different intercropping systems. In vitro infection tests were carried out to reveal the effect of the CLE on soybean pod resistance to mildew infection. CFI and metabolomics preliminarily revealed the mechanism of improved mildew resistance in soybean pods

growing under a CLE that simulated from an MSI system.

2 MATERIALS AND METHODS

2.1 Plant materials and experimental design

A conventional soybean cultivar C103, which was provided by a specialist company in the region and was used for this study, was grown at the farm of Sichuan Agricultural University in Ya'an, Sichuan Province, China (29°59′N, 103°00′E), in 2018 and 2019. A single-factor random block design was applied to soybean plants in the wide-narrow row planting mode (figure 1), and 4 treatments, including whole growth stage shading (WS), vegetative stage shading (VS), reproductive stage shading (RS), and whole growth stage normal light (WL), were applied over 12 plots (figure 1). Soybean pods were harvested, cleaned, and inoculated at the R5 (beginning seed) stage, including uninoculated (CK) and *Fusarium verticillioides* (Fv)-inoculated pods, and each treatment was repeated for 5 independent biological replicates. CFI parameters and the mildew index were used to test Fv resistance. From 7 DAI (days after inoculation), soybean pods were frozen in liquid nitrogen and stored at -80°C. Metabolites were qualitatively and quantitatively analyzed by ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS); data were analyzed by multivariate statistics, such as principal component analysis (PCA). The main workflow is shown in figure 1.

2.2 Fungal inoculation

The *F. verticillioides* strain was isolated and identified from field-grown soybean pods in

our previous study(Liu et al., 2017). The strain was cultured at 30 °C overnight in PDA medium on a rotary shaker. Then, the culture medium was centrifuged for 10 min at 3000 rpm, and the supernatant was discarded. The conidia were washed 3 times, counted with a hemocytometer, and adjusted to a density of 10^6 cfu·ml^{-1}.

Full, healthy soybean pods were washed 2-3 times with sterile water, and the surface was wiped clean. To facilitate fungal infection, the surface of each soybean pod was punctured into 3 portions using a sterile needle, and each surface-damaged portion was inoculated with a conidial suspension of *F. verticillioides* (5 μL, 10^6 cfu·ml^{-1}) or sterile distilled water (as a control). The inoculated pods were placed in an incubator at 30 °C for 7 d.

2.3 Mildew survey and resistance evaluation

Soybean pods were used to test the resistance to fungal infection with the mildew index, which classifies infections into 5 different levels: 0 level (no lesions), 1st level (the inoculated area shows a few white hyphal strands), 2nd level (the lesioned area≤1/4 of a single pod's total surface area), 3rd level (1/4 of a single pod's total surface area < lesioned area ≤1/2 of a single pod's total surface area), 4th level (1/2 of a single pod's total surface area < lesioned area ≤3/4 of a single pod's total surface area) and 5th level (lesioned area > 3/4 of a single pod's total surface area). The lesioned area was measured by visual inspection. The mildew index and mildew rate were calculated as described in our previous study: Mildew index =

$\frac{\sum p_i c_i}{n c_{max}} \times 100$; Mildew rate = $\frac{f}{n} \times 100$ (p_i: number of molded pods of various grades; c_i: corresponding infection grades; n: number of pods surveyed; c_{max}: top infection grade; f: the sum of p_i;).

The maximum quantum yield of photosystem II (*Fv/Fm*) and photochemical quenching (*qP*) in soybean pods were detected by a chlorophyll fluorescence imager (British Technologica company) at 1, 2, 3, 4, 5, 6, and 7 DAI. *Fv/Fm* reflects the potential maximum light energy conversion rate in plants and reveals the adaptability of plants to light intensity in the environment. *qP*, which is the photochemical quenching caused by increasing photosynthesis, reflects the efficiency of light energy conversion to photosynthetic assimilates(Rousseau et al., 2013).

2.4 Exogenous JA inhibitor treatment

Three experimental treatments, including fungal inoculation (F), spraying with the lipoxygenase inhibitor phenidone (PHD) before inoculation (PF), and water application as a control (CK), were applied in this section. In brief, three groups of healthy uniform soybean pods were selected for the test; 100 μM PHD was sprayed on the pod surfaces of the PF group, and the pods of the other two groups, F and CK, were sprayed with the same amount of sterile water. After 30 min, all the groups were inoculated with 10 μL of 10^6 cfu·ml^{-1} spore solution (containing 0.01% Tween solution). Treated soybean pods were collected after 0 h, 12 h, 24 h, 48 h, and 84 h and frozen in liquid nitrogen immediately. All the samples were stored at -80°C for further analysis.

2.5 Gene expression analysis

Total RNA from soybean pods was extracted using a TIANGEN RNAprep Pure Plant Kit (TIANGEN Biotech (Beijing) Co., Ltd.) according to the manufacturer's instructions. The concentration and purity of total RNA from the samples were determined by a NanoVue plus instrument. Complementary DNA (cDNA) was synthesized using HiScript® II Q RT SuperMix for qPCR (+gDNA wiper) following the manufacturer's (Vazyme Biotech Co., Ltd.) protocol. RT-qPCR of diluted template cDNA was carried out using ChamQ™ Universal SYBR® qPCR Master Mix (Vazyme Biotech Co., Ltd.) as described the manufacturer's instructions. The reaction system (10 μL) comprised diluted template cDNA (1 μL), ddH_2O (3.6 μL), forward and reverse primers (0.2 μL, respectively), and 2 × ChamQ Universal SYBR qPCR Master Mix (5 μL). RT-qPCR was performed on cDNA from three biological replicates by a fluorescence quantitative PCR instrument (Life Technologies, QuantStudio6 Flax); the primers used in this study are listed in Supplemental Table S1.

2.6 Metabolomics analysis

2.6.1 Sample extraction and preparation

The 7 DAI soybean pods were ground with liquid nitrogen and lyophilized for 48 h using a vacuum freeze dryer. Approximately 20 mg pod powder from each replicate was transferred into a 2 mL precooled centrifuge tube, and 1 mL 80% methanol extraction solution was added. The samples were mixed for 10 s and extracted at 4 °C for 1 h in an ultrasonic water bath (40 kHz, 300 W). The samples were centrifuged at 11,000 g and 4°C for

10 min, and then the supernatant fluid (approximately 800 μL) was filtered into a sample bottle through a syringe filter (0.22 μm) and injected directly into a UPLC-MS system.

2.6.2 Untargeted metabolomics analysis by UPLC-Q-TOF/MS

Untargeted metabolomics analysis was carried out by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS); the UPLC system was a 1290 Infinity from Agilent Technologies, and the MS was a 6545 Q-TOFMS. Approximately 1 μL of each separated sample was separated with an Agilent Eclipse Plus-C18 column (100 × 2.1 mm, 1.8 μm), and the column temperature was 35 °C. The mobile phase was composed of eluent A (0.1% formic acid aqueous solution) and eluent B (0.1% formic acid acetonitrile solution), and the flow rate was set as 0.300 $ml \cdot min^{-1}$. Gradient elution was as follows: 0 to 4 min, 85 to 78% eluent A; 4 to 10.5 min, 78 to 61% eluent A; 10.5 to 13 min, 61 to 56% eluent A; 13 to 17.5 min, 56 to 0% eluent A; 17.5 to 20 min, 0 to 85% eluent A. For mass spectral conditions, positive ion mode was used. Nitrogen was used as the collision gas; the ESI sprayer pressure was 20 psig, and the MS-TOF fragment pressure was 140 V. The desolvation gas flow rate and temperature were 10 $L \cdot min^{-1}$ and 325 °C, respectively. The autosampler temperature was maintained at 4 °C. In addition, full scan mode was used for mass spectral acquisition, and the scanning frequency was 1 s/spectra.

2.6.3 Targeted quantitation of isoflavones

The isoflavones in soybean pods were quantified using ultra-performance liquid chromatography-triple quadrupole mass spectrometry (UPLC-MS/MS, Waters Xevo TQ-

Smicro). The main chromatographic conditions were as follows: chromatographic column, ACQUIY UPLC BEH-C18 column (50 mm×2.1, 1.7 μm); column temperature, 35 °C; mobile phase, A (0.1% formic acid aqueous solution) and B (0.1% formic acid acetonitrile solution); gradient elution, 85-75% A (0-4.8 min), 75-60% A (4.8-7.2 min), 60-85% A (7.2-7.23 min), 85% A (7.23-9 min); injection volume, 1 μL; flow rate, 0.350 $ml \cdot min^{-1}$. The mass spectra were measured under the following conditions: electrospray ionization (ESI); positive ion mode; desolvation temperature, 350 °C; desolvation volume, 10 $L \cdot min^{-1}$; cone voltage, 35 kV. Nitrogen was used in the ion source and as the collision gas, and the collision voltage was 3 kV. The main isoflavones in soybean pods were quantified in absolute terms via linear regression of their corresponding standards.

2.7 Statistical analyses

The raw chromatographic peaks obtained from UPLC-QTOF-MS were uploaded to XCMS Online (https://xcmsonline.scripps.edu/) and subsequently deconvolved, which included peak recognition, peak filtering, peak alignment and more. The final results included a two-dimensional data matrix after deconvolution (m/z, retention time, *P*-value, *q* value, peak area), differential feature ion compound matching, and metabolic pathway annotation. MetaboAnalyst 4.0 (https://www.metaboanalyst.ca/) was used for PCA, heatmap, and pathway enrichment analyses. Quantification data were subjected to correlation, regression, and variance analyses with SPSS.

3 RESULTS

3.1 Mildew resistance evaluation

To assess the fungal resistance of soybean pods grown under a changing light environment (CLE), *F. verticillioides* was inoculated onto the pod surface; chlorophyll fluorescence parameters and mildew indexes were monitored over 7 d in an incubator (figure 2). As shown in figure 2a, chlorophyll fluorescence imaging of soybean pod lesions was significantly affected during fungal infection. After inoculation, the fluorescence color of the inoculated portion of the soybean pods changed from red to green to dark blue, which showed that the infection degree increased (figure 2a). The changed portions of some pods appeared blue at 3 to 4 DAI in the WL-Fv and WS-Fv groups. At 5 DAI, the inoculated spots of some pods in the RS-Fv group also became blue. At 7 DAI, the inoculated spots of all pods in WS-Fv turned dark blue, which indicated tissue necrosis, while the inoculated spots of only a few of the pods appeared dark blue in the VS-Fv group.

The chlorophyll fluorescence parameters also changed as indicated by the fluorescence imaging in inoculated soybean pods. The *Fv/Fm* (optimal photochemical efficiency of PSII in the dark) ratio of all pods decreased with the DAI (figure 2b), and the *Fv/Fm* ratios of pods in WS-Fv significantly declined (by 0.5387 from 1 d to 7 d). However, the *Fv/Fm* ratio of all pods in the VS-Fv group merely decreased by 0.1888. Another important fluorescence parameter, the *qP* (photochemical quenching) value of pods, was enhanced with DAI but not significantly, except in the WS-Fv group (figure 2c); however, the *qP* value of the WS-Fv group was significantly higher than that of the other groups at 7 DAI, and the VS-Fv group had the lowest qP value. Compared with those at 1 d, the *qP* values of infected pods at 7 d were increased by 0.8202, 0.2312, 0.1841, and 0.1047 for the WS-Fv, WL-Fv, RS-Fv, and

VS-Fv groups, respectively (figure 2c).

Similar to the changes in the *qP* values, the mildew index of soybean pods rose with the DAI. Although the macroscopic mildew index was not more accurate than the chlorophyll fluorescence parameters, it was clear that the mildew indexes of the VS-FV and RS-FV groups were lower than those of the other two groups (figure 2d). As shown in figure 2e, the average mildew indexes of distinct groups at 7 d were sorted: VS-Fv (20.98) < RS-Fv (25.42) < WL-Fv (30.25) < WS-Fv (31.85). Combined with the CFI and mildew index, these results suggest that the mildew resistance of pods growing under changing light conditions (VS-Fv and RS-Fv) was higher than that of pods growing under uniform light conditions (WL-Fv and WS-Fv), especially for pods subjected to shading at the vegetative stage (VS-Fv).

3.2 Untargeted metabolomics analysis

3.2.1 Metabolic profiling and clustering

According to the CFI and mildew index analyses, the mildew resistance of soybean pods growing under different light environments exhibited significant disparities. To further reveal the important metabolites in soybean pods that contribute to mildew resistance, a comparative metabolomic analysis among all treatments was conducted by UPLC-Q/TOF-MS. The PCA results showed that the metabolic profiling of soybean pods was affected by light conditions and fungi (figure 3a). In particular, as shown in figure 3a, the Fv-inoculated (right side) and uninoculated groups (left side) were considerably separated by PC1 (50.9%), which showed that mold dramatically affected the pod metabolites. Although all *Fv*-inoculated groups were clustered together, the VS-Fv group (blue area) was separated from the other groups, and it

was especially separated from the WS-Fv group (orange area) and WL-Fv group (yellow area). This regular phenotype was confirmed from the cluster analysis; as shown in the heat map of figure 3b, the RS-Fv and WL-Fv groups were clustered together and close to the VS-Fv group. A red area in the top clustering of the VS-Fv group is quite prominent, demonstrating that the contents of some metabolites were increased significantly compared to those of other groups; these upregulated compounds in VS-Fv pods might play an important role in pathogen resistance.

3.2.2 Pathway enrichment analysis

To explore the potential resistance mechanism in soybean pods, the different metabolites screened by VS-Fv and WL-Fv (as controls) were imported into the KEGG pathway database for pathway annotations. The pathway enrichment analysis showed that there were significant differences in phenylpropanoid and flavone metabolic pathways, including kaempferol glycoside biosynthesis, quercetin glycoside biosynthesis, flavonoid biosynthesis, coumarin biosynthesis, flavonol biosynthesis, leucodelphinidin biosynthesis, phenylalanine biosynthesis, luteolin biosynthesis, and wogonin metabolism (figure 4). In particular, as shown in figure 4, quercetin glycoside biosynthesis, kaempferol glycoside biosynthesis, flavonoid biosynthesis, and flavonol biosynthesis were significantly upregulated in the VS-Fv group compared with the WL-Fv group, in which 13, 12, 8, and 5 metabolites were annotated, respectively. The significant increase in flavonoid and flavonol contents in soybean pods of the VS-Fv group suggested that these metabolites might play a key potential resistance function.

Moreover, the JA biosynthesis pathway was also associated with the VS-Fv group (as shown in figure 4 in blue). Abundant evidence has confirmed the antifungal activities of flavonoids in plants(Weston & Mathesius, 2013); it is well known that JA is frequently induced by biological stress and plays an important role in plant immune and defense responses(Jang, Yoon, & Choi, 2020). Hence, according to the pathway enrichment analysis, JA and flavonoids could be involved in the VS-treated pod defense response and may enhance mildew resistance.

3.3 Targeted analysis of isoflavones

Isoflavones are the major flavonoid metabolites in soybean plants. Our previous research confirmed the significance of isoflavones to the mold resistance of seed pods(Liu, Deng, et al., 2016). Combined with the above metabolomics analysis, which indicated that the flavonoid synthesis pathway was significantly enriched in phenylpropane metabolism (figure 4), we hypothesized that isoflavone and downstream glyceollin biosynthesis was a key response to mold infection(Sukumaran, McDowell, Chen, Renaud, & Dhaubhadel, 2018). Hence, the isoflavones of soybean pods were quantitated by UPLC-MS/MS and annotated into metabolic pathways by using heat maps (figure 5). As shown in figure 5, almost all the isoflavone contents in the noninoculated pods were lower than those in the inoculated pods; mold inoculation can induce isoflavone biosynthesis in soybean pods. Detailed comparison among the non-inoculated pods (all CK-marked groups) showed that the contents of aglycone and glucoside in pods growing under CLE (VS-CK and RS-CK) were higher than those in the WL-CK group except for glyceollin I and glycitein (figure 5), which were suggested as

potential resistance inducing compounds(Sahu et al., 2017).

On the other hand, the contents of upstream aglycones, such as liquirigenin, naringenin, genistein, glycitein, and daidzein, were not significantly different between the WL-Fv and RS-Fv groups but were significantly downregulated in the VS-Fv group compared to the WL-Fv group. The contents of isoflavone glycosides and glyceollin, which are located downstream of the isoflavone biosynthesis pathway, were significantly higher in VS-Fv than in WL-Fv, especially isoflavone glucoside (genistin, glycitin, and daidzin), malonyl-glucoside (malonylgenistin, malonylglycitin, and malonyldaidzin), and glyceollin I.

These results suggested that *F. verticillioides* could induce isoflavone metabolism flow downstream to synthesize malonyl-glucoside and glyceollin I, resulting in a faster chemical defense response of soybean pods. The enhancing effect of CLE on the upstream aglycones further provides enough aglycone precursors for the synthesis of induced resistance compounds, including malonyl-glucoside and glyceollin, eventually leading to an increase in the resistance of pods growing under a CLE.

3.4 Correlation analysis of JA and isoflavones

Enrichment analysis of different metabolites showed that both JA and flavonoid biosynthesis in soybean pods were significantly affected by VS-Fv (figure 4). The quantitative results also suggested that the malonyl-isoflavone contents in the VS-Fv group increased markedly after induction by *F. verticillioides* (figure 5). To reveal the relationship between JA and malonyl-isoflavones, the metabolomics data of inoculated pods (all Fv-marked groups) were used for correlation analysis. According to the exact mass of ion

features and retention times, the ionic strength of JA and malonyl-isoflavones were extracted from the metabolomic data matrix. Two ionic derivatives of JA, which were elucidated as $JA+H^+$ and $JA-H_2O+H^+$, were used for correlation analysis (Supplemental Figure S1).

The linear regression analysis of the ionic strength of JA derivatives and malonyl-isoflavones (MG, MGL, and MD) indicated that malonyl-isoflavones increased with the JA content (figure 6). Here, the ionic strengths of $JA+H^+$ and $JA-H_2O+H^+$ were both significantly positively related to the contents of MG, MGL, and MD with high correlation coefficients. Simultaneously, the ionic strength of JA and three malonyl-isoflavones in the VS-Fv group were the highest, followed by those of the RS-Fv and WL-Fv groups, while those of the WS-Fv group were the lowest. This phenotype tends toward Fv resistance in seed pods harvested from soybean plants growing under a CLE. The above results suggested that JA and malonyl-isoflavones are synergistically vital during resistance to *F. verticillioides* infection in soybean pods.

3.5 Effect of JA on soybean pod flavonoid responses to mildew infection

3.5.1 Mildew resistance phenotype of soybean pods treated with exogenous JA inhibitor

To further confirm the effects of JA on isoflavone biosynthesis in soybean pods, an exogenous PHD application test was conducted in vitro. PHD is an inhibitor of JA biosynthesis that blocks endogenous JA synthesis by inhibiting lipoxygenases(Patkar et al., 2015). The mildew resistance phenotypes of soybean pods at 84 hpi are shown in figure 7. Fungal inoculation induced taupe fungal plaques covered with white hyphae, which could

reflect the mildew degree. There were significantly more plaques on PHD-treated pods (PF) than on non-PHD-treated pods (F) (figure 7a). The mildew index and mildew rate of the PHD-treated pods were significantly higher than those of pods without PHD application (figure 7b). This result indicated that PHD-treated soybean pods were more susceptible to fungal infection and that JA had a positive impact on the mildew resistance of pods.

3.5.2 Inhibitory activity of PHD on flavonoid accumulation in soybean pods

Flavonoids are the components involved in antifungal activity in legumes. JA signaling is likely to regulate flavonoid synthesis in soybean pods to affect the mold defense response. According to the time sequence monitoring of major flavone constitutions in soybean pods with different treatments, all flavonoid contents in the inoculated pods were significantly higher than those in the control groups (CK) within 12-84 h of infection. However, PHD application led to a decrease in flavonoid levels in soybean pods, which were almost the same as those in the CK groups (figure 8a). Detailed quantitative analysis of the inoculated pods without PHD application was carried out, especially comparing the CK and F groups. The results showed that some chemicals in the upstream pathway responded to mildew early, including GE, DE, GEG, formononetin, liquiritigenin, and luteolin; their changing curves were immediately different from those of the control groups within 12 h after inoculation and showed the highest contents at 24 h or 48 h. While other chemicals in the downstream pathway, such as MG, MD, AD, DG, GLG, glyceollin I, glyceollin V, and glyceofuran, responded later, within 24 h after inoculation, their accumulation continued to increase with the infection duration (figure 8a). However, the flavonoid contents decreased drastically

when PHD was applied. In particular, glyceollin I and glyceofuran are recognized as antifungal chemicals in legumes. The above two glyceollins were seriously inhibited by PHD, and their accumulation decreased by up to 7.9 times and 11.3 times, respectively, compared to that in pods receiving inoculation only (F), while some aglycones, such as GLE and formononetin, were less affected by PHD application (figure 8a).

Moreover, the expression patterns of key genes involved in isoflavone biosynthesis in pods after 5 h of treatment were quantified by qPCR. *GmUGT* and *GmMT7* are the key enzyme genes closely related to downstream isoflavone glycosylation and malonylation; *GmG4DT* and *GmGS* are genes closely related to glyceolin biosynthesis. As shown in figure 8b, the expression levels of *GmUGT*, *GmMT7*, *GmG4DT*, and *GmGS* were significantly higher in the inoculated groups (F) than in the CK group, while the upstream *GmIFS* expression was not significantly different. However, after spray application of PHD, the expression levels of the above key genes in the PF groups were downregulated significantly compared with those in the inoculation-only group (F), in which *GmUGT GmMT7*, *GmG4DT*, and *GmGS* were downregulated by 64.95%, 66.74%, 97.18%, and 98.65%, respectively (figure 8b). This suggests that PHD could inhibit the expression of pod isoflavone-related genes by repressing JA signaling in response to *F. verticillioides*. Additionally, the above results also reveal that the JA signaling pathway mainly affects special malonyl-isoflavones and glyceollins, which are downstream of the flavonoid biosynthesis pathway.

4 DISCUSSION

4.1 Soybean pods play an important role in pathogen response

Seedpods are the outermost barrier of legumes against pests and diseases and play an important role in physical and chemical defense. Previous studies have focused more on the resistance of soybean seeds and less on that of bean pods(Deng et al., 2017). Some researchers suggest that nonglandular trichomes on the surface of bean pods are epidermal appendages that mechanically capture *Liriomyza trifolii*(Xing et al., 2017) and resist the feeding and oviposition of herbivores such as stink bugs(Lam & Pedigo, 2001). In addition to physical defense, bean pods also play chemical defense functions: constitutive isoflavone conjugates and UV-induced isoflavones can prevent the invasion of stink bugs in field-grown soybean(Zavala, Mazza, Dillon, Chludil, & BallarÉ, 2015), and isoflavone and glyceolin from green vegetable soybean have excellent antimicrobial activities(T. Wang et al., 2018). Our previous studies also indicated the physical barrier effect of the soybean seed pod, which reduced the damage to soybean seeds from field mold and revealed a protective effect against mold infection(Liu et al., 2017). Moreover, we screened and obtained different soybean germplasms with organ-specific mildew resistance and further confirmed the important relationship between the isoflavone content in the seed pod and mildew resistance(Liu, Deng, et al., 2016). Therefore, the bean pod is the first line of defense against pests and diseases in legumes and can be used as a phenotypic indicator to characterize the biological stress resistance of soybean germplasm.

Biological stresses such as insect damage and pathogen infection can change the photosynthetic metabolism of infected plant parts and influence the chlorophyll fluorescence yield of green tissues(Rolfe & Scholes, 2010). Therefore, chlorophyll fluorescence imaging

(CFI), which is a noninvasive tool to monitor photosynthetic performance in vitro, can be used as a potential phenomics technique for detecting the level of fungal infection(Scholes & Rolfe, 2009)**.** In our current study, CFI was used for the first time to evaluate the resistance of soybean pods after inoculation in vitro. The CFI evaluation results were highly consistent with the classical subjective method but more efficient and visualizable (figure 2). In particular, the use of fluorescence parameters such as *Fv/Fm* and *qP* made the evaluation results more accurate and reliable, which greatly improved the accuracy and efficiency of resistance evaluation.

4.2 Promotion of isoflavone synthesis in the seed pod by changing light partially unveils the mystery of higher resistance of intercropped soybean seeds

Intercropping is an effective measure to improve land utilization; reasonable intercropping can partially protect crops from pathogen and herbivore attacks(Li et al., 2020). Maize-soybean intercropping (MSI) is an ecological, efficient, and sustainable cultivation mode that is widely used in developing countries and has recently attracted much attention in developed countries(Gao et al., 2017). MSI has multiple ecological effects, such as reducing pests and diseases; elucidation of the mechanism of action will provide an important theoretical basis for further optimizing cultivation measures and improving crop yield and quality. However, we still know little about this so far. Although most of the previous studies focused on the inhibitory effect of biodiversity on pests and diseases, few studies have examined the mechanism of crop resistance enhancement mediated by the abiotic environment, especially the light environment(Brooker et al., 2015). The novelty of

this study came from the examination of an actual field MSI system, in which the maize's higher height resulted in shading for the soybean plant(WANG Yi, 2016).

In legumes, flavonoids are known to play pivotal roles in response to biotic and abiotic factors. The current results indicated that light conditions and mold inoculation both affect metabolic profiling. The PCA score plots and clustering analyses suggested that fungal infection had a greater effect on the pod metabolome than light treatment (figure 3). Plant disease resistance can be divided into two types: constitutive resistance (CR) and induced resistance (IR). CR is an inherent characteristic of plants before they are harmed by stresses; it is produced from phyletic evolution over a long time and determined by plant genotype. Plant CR is also affected by environmental factors(Kempel, Schädler, Chrobock, Fischer, & van Kleunen, 2011). IR refers to the resistance components or reactions induced by the pathogen or herbivore(Anne, Martin, Thomas, Markus, & Mark, 2011). The effect of abiotic light conditions on the plant metabolome is sustained for a long time and is mainly reflected in the accumulation of constituent metabolites. However, the response of plants to fungal infection is usually quick, and some reactions are even instantaneous. The reaction during this stage is mainly reflected in the accumulation of inductive metabolites. Additional studies also confirmed that there are tradeoffs between CR and IR (Kempel et al., 2011). In this study, there was a tradeoff in the metabolic flow conversion among isoflavones with different structural types as their core. The synergy of the CR isoflavone metabolite aglycone and the IR isoflavone metabolites glycoside and glyceollin improved the resistance of soybean pods.

Many studies have shown that flavonoid biosynthesis is a light-dependent carbon fixation process(Nam, Lim, & Eom, 2018). Better light conditions are beneficial to the

synthesis and accumulation of flavonoids in plant tissue, especially high-intensity and shortwavelength light irradiation (such as ultraviolet and blue light), which can improve flavonoid biosynthesis(Siipola et al., 2015). Soybean isoflavone, as an important type of plant flavones, is no exception; many pieces of research have demonstrated that high latitude and long days are beneficial to the synthesis and accumulation of isoflavone in soybean seeds(H.-j. Wu et al., 2017). In the field MSI system, maize leaves do not always shade soybean plants. In particular, soybean is shaded by maize mainly during the vegetative stage (VS) in the relay intercropping system. After maize harvest, the light conditions of the soybean canopy are improved greatly. Therefore, the light condition in this MSI system has the characteristic of "changing from weak to bright" with spatiotemporal heterogeneity(Dennis et al., 2020).

Previous studies have focused on the homogeneous light environment, namely, the influence of continuous stable light conditions; however, less attention has been given to the heterogeneous light environment. Yushan Wu et al. researched the physiological responses to shade and subsequent recovery of soybean in an MSI system and indicated that the illuminance of the soybean canopy was recovered after maize harvest; soybean leaf area and leaf mass increased even more than those of monocropping soybean(Y. Wu et al., 2016). The compensatory growth of intercropped soybean plants in the reproductive stage is important and provides carbon sources for the growth of reproductive structures and the biosynthesis of secondary metabolites.

Our recent study showed that the isoflavone contents of soybean seeds were significantly higher in MSI than in monocropping systems(Liu, Yang, et al., 2016). We

therefore speculated that vegetative stage shading (VS) could also improve isoflavone accumulation in soybean seedpods and enhance tolerance to pathogens. The enrichment analysis of different metabolites (figure 4) and targeted flavonoid quantification (figure 5) indicated that phenylpropanoid metabolism in pods, especially flavonoid biosynthesis, was significantly upregulated by VS. These current results confirmed that CL conditions, especially VS, could improve the resistance of soybean seedpods to *F. verticillioides*. Hence, the increase in isoflavone contents in soybean pods under a changing light environment was one mechanism of the enhancement of soybean resistance by intercropping.

4.3 JA-mediated responses to the combined stress of shading and mildew triggered potential cross-resistance in soybean seedpods

In nature or agricultural practice, plants are often affected by multiple stresses, which is defined as combined stress. The effects of combined stress on crops are not always disadvantageous and are determined by the interactive mechanisms between stress factors. More studies have shown that abiotic stresses such as drought, high/low temperature, and salt stress can affect the occurrence and spread of pathogenic bacteria, insects, and weeds. These stresses directly or indirectly regulate plant-pest/disease interactions by altering plant physiology and related physicochemical defense responses. Multiple individual stresses are special combined stresses that refer to two or more stresses that do not occur in the same period, namely, one stress after another with no overlapping effect on plants (P. Pandey, Irulappan, Bagavathiannan, & Senthil-Kumar, 2017). This is also called sequential stresses. Under the MSI pattern, the soybean plants experienced maize shading in the vegetative stage

503 and fungal infection in the late reproductive stage, which is similar to an abiotic-biotic
504 sequential stress. Our results confirmed the beneficial regulatory effects on soybean pods
505 from the abovementioned sequential stresses. This advantage effect can be called “cross-
506 resistance” (Foyer, Rasool, Davey, & Hancock, 2016). Phytohormone signaling networks are
507 actively stimulated by diverse environmental factors(J. Yang et al., 2019). The synergistic
508 effect of endogenous hormones and secondary metabolites is an important mechanism of
509 cross-resistance in plants under combined stress. Prior stress may either lead to priming or
510 predisposition for plant metabolite biosynthesis during a subsequent stress (Prachi Pandey,
511 Ramegowda, & Senthil-Kumar, 2015).

512 Recent research on pod resistance to insects indicated that herbivory- and solar UV-B
513 radiation-induced increases in the level of defensive isoflavones in pods against stink bugs
514 were mediated by ethylene signaling(Dillon, Chludil, Mithöfer, & Zavala, 2020). Another
515 well-known factor, JA, is a typical biological stress hormone that plays a key role in plant
516 disease resistance(Jang et al., 2020). This research indicated that the CL environment (VS
517 and RS) did not have a positive effect on JA biosynthesis in healthy soybean pods. Fungal
518 inoculation increased the JA content and significantly induced isoflavone synthesis, which
519 was consistent with existing research results(Jeong et al., 2018; Lozovaya et al., 2004).
520 However, even more importantly, JA biosynthesis was significantly increased by mold
521 infection in the VS group compared to the WL group growing under normal light conditions
522 (figure 3). Namely, after Fv inoculation, the JA response difference between the VS and WL
523 groups was greater than that of the soybean pods without Fv inoculation. Additionally, the
524 exogenous JA biosynthesis inhibitor PHD also induced a phenotype of increased mildew

susceptibility compared with the blank control (CK) and inoculation (F) treatments (figure 7). Previous studies have revealed that biological stress, such as pathogen infection and insect damage, can induce JA signaling *in planta*. JA activates flavonoid biosynthesis downstream of the phenylpropanoid metabolic pathway, improving defense ability (Gaquerel, Gulati, & Baldwin, 2014; Min et al., 2020). In this study, JA showed a significant positive correlation with isoflavone (figure 6), and the isoflavone contents were significantly reduced after PHD spraying (figure 8). All the above studies indicated that JA could defend against mold infection by positively regulating the accumulation of isoflavone in soybean pods, which can be promoted by VS.

Chico et al. proposed a JA signaling model: in a non-induced situation (without JA), the transcription factor MYC2 is repressed by direct binding to the JAZ protein. Upon external stimulation, SCF^{COI1} targets JAZ repressors for proteasome-dependent degradation in response to JA and then releases MYC2 and other active TFs. MYC2 further activates downstream resistance genes(Chico, Chini, Fonseca, & Solano, 2008). Similarly, interaction analysis by chemical association networks (http://stitch.embl.de/cgi/input.pl) revealed that JA regulates flavonoid biosynthesis mediated by the MYB transcription factor (Supplementary figure 2)(Y. Wang et al., 2019). Recent similar work has also identified an additional transcription factor, *GmMYB29A2*, which regulates soybean glyceollin biosynthesis and mediates resistance to *Phytophthora sojae* (Jahan et al., 2020).

Isoflavone quantitative analysis and JA exogenous inhibitor tests both indicated that endogenous JA signaling was activated by mildew infection of pods; its regulatory effect on downstream isoflavone biosynthesis was more obvious than that of the corresponding

upstream effect. However, almost all MYB transcription factors discovered until now act on the upstream structural genes of flavonoid synthesis, such as *CHS*, *IFS*, and *CHI*(Garcia-Calderon, Perez-Delgado, Palove-Balang, Betti, & Marquez, 2020). Therefore, in combination with this current study, we believe there are latent transcription factors that regulate downstream genes such as *UGT* and *MT* involved in isoflavone synthesis in soybean pods. This remains to be further explored.

In conclusion, we can summarize the mechanism by which soybean pod resistance improves mildew infection under a CLE or relay intercropping systems as follows (figure 9): fungal infection activates JA signaling in soybean pods and induces the biosynthesis of malonyl-isoflavone and glyceollin. Beforehand, the photosynthetic carbon fixation capacity of pods was enhanced by a CLE, which provided sufficient precursors for the biosynthesis and accumulation of downstream isoflavones and then improved the defense function of seed pods.

Mechanistic research on soybean pod resistance to mildew improved by a CLE (especially VS) provides a theoretical basis for soybean disease resistance via intercropping. Research on the strengthening of soybean stress tolerance has also opened up new directions. Furthermore, it is expected that important gene resources will be discovered from soybean pods to lay the foundation of resistance breeding by molecular tools. So far, many questions remain; for example, how shading during the vegetative stage and reillumination during the reproductive stage promote the biosynthesis and accumulation of isoflavone precursors is not fully understood.

According to the existing literature and our recent research, two scientific hypotheses

can be proposed: 1- a special CLE (weak to bright light conditions) leads to the photoinhibition of soybean leaves, in which excess excitation energy causes illumination injury in cellular organelles(Zhou, Su, Zhang, Zhang, & Guo, 2016), while biosynthesis isoflavone, as a strong antioxidant chemical component, is a photoprotective mechanism initiated by soybean plants (Mazza et al., 2000). 2- VS partially inhibited canopy growth and weakened the "self-shading" effect on soybean pods during the maturation stage; this cultural technique improved photosynthetic and carbon fixation, and the biosynthesis and accumulation of soybean isoflavone was promoted(Collison, Raven, Pignon, & Long, 2020). In these respects, we have obtained certain evidence for the mechanisms of increased resistance via intercropping, but further research is still needed.

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CONFLICT OF INTEREST

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

Jiang Liu and Wenyu Yang designed the research. Xiaoman Li, Caiqiong Yang, Juncai Deng, Congwei Xie, Xinli Xiao, and Xiyang Long performed the experiments. Xiaoling Wu,

Weiguo Liu, Junbo Du, Feng Yang, Xiaochun Wang, Taiwen Yong, Jing Zhang, and YuShan Wu assisted the experimental design, analyzed and discussed the data. Xiaoman Li and Jiang Liu wrote the manuscript with contributions from all the co-authors. All authors approved the final version of the manuscript.

SUPPLEMENTAL DATA:

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Key gene primers.

Figure S1. The ion strength, structural formula, and mass spectrometry information of JA derivatives.

Figure S2. The interaction analysis of JA and the flavonoid metabolism network by STITCH: chemical association networks (http://stitch.embl.de/cgi/input.pl).

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FIGURE LEGENDS (main text)

FIGURE 1. Experimental design and workflow.

FIGURE 2. Comparison of mildew resistance of soybean pods. Chlorophyll fluorescence imaging of pods showing a mildew gradient (a), *Fv/Fm* (b), *qP* (c), mildew index (d), average mildew index (e).

FIGURE 3. PCA score plots (a) and clustering heatmap (b) of mold-infected soybean pods growing under different light conditions.

FIGURE 4. Pathway analysis of mold-infected pods growing under different light conditions (VS-Fv vs. WL-Fv). The color and size of pathway symbols represent the significance level in the enrichment analysis and the impact factor, respectively.

788

789 **FIGURE 5. Integrated effects of shading and mold infection on isoflavone biosynthesis**
790 **in soybean pods.** The isoflavone contents in soybean pods under different treatments are
791 represented by colors ranging from blue (low) to red (high) for each compound.

792

793 **FIGURE 6. Correlation analysis of JA and isoflavone contents in soybean pods.** R^2: The
794 determination coefficient (squared Pearson correlation coefficient) represents the imitative
795 effect of the linear regression equation; **: Correlation is significant at the 0.01 level (2-
796 tailed). MG: malonylgenistin, MGL: malonylglycitin, MD: malonyldaidzin.

797

798 **FIGURE 7. Effects of JA synthesis inhibition on the mildew resistance phenotypes of**
799 **soybean pods.** CK: sterile water control; F: inoculation with *F. verticillioides*; PF: PHD
800 pretreatment + fungal inoculation

801

802 **FIGURE 8. Effect of phenidone on flavonoid biosynthesis in soybean pods.** (a) Dynamic
803 changes in flavonoid accumulation; (b) Expression analysis of key genes involved in
804 isoflavone biosynthesis. CK: sterile water control; F: inoculation with *F. verticillioides*; PF:
805 PHD pretreatment + fungal inoculation. GE: genistein, GLE: glycitein, DE: daidzein, GEG:
806 genistin, GLG: glycitin, DG: daidzin, AD: acetyldaidzin. *GmIFS2*: isoflavone synthase;
807 *GmUGT*: glycosyltransferase; *GmMT7*: malonyltransferase; *GmG4DT*: glycinol 4-
808 dimethylallyl transferase; *GmGS*: glyceolin synthase.

809

810 **FIGURE 9. A proposed model for the mechanism by which the combination of changing**

light and endogenous JA enhance mildew resistance in soybean pods.

CLE (VS) increases the level of carbon assimilation, providing an adequate carbon source for the biosynthesis of upstream aglycones and glucosides. In response to fungal infection, the JA signaling pathway of pods was activated; the upregulated *MYBs* induced the biosynthesis of downstream flavonoids and further enhanced the mildew resistance of soybean pods.