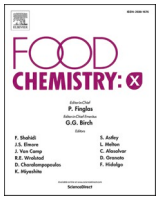
Food Chemistry: X 14 (2022) 100331

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



UV-B- triggered H_2O_2 production mediates isoflavones synthesis in germinated soybean

Meng Ma [a,b,1], Wenlin Xu [a,1], Pei Wang [b], Zhenxin Gu [b], Hongzhi Zhang [c,*], Runqiang Yang [b,*]

[a] College of Food Science and Engineering, Qingdao Agricultural University, Qingdao, Shandong 266109, People's Republic of China
[b] College of Food Science and Technology, Nanjing Agricultural University, Nanjing, Jiangsu 210095, People's Republic of China
[c] Institute of Agro-Product Processing, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu 210095, People's Republic of China

ARTICLE INFO

Keywords:
H_2O_2
UV-B
Germinated soybean
Isoflavones

ABSTRACT

In this study, the functions of Hydrogen peroxide (H_2O_2) on the synthesis of isoflavones in germinated soybean under UV-B radiation were investigated. Results showed that the activity, gene, and protein expression of NADPH oxidase were up-regulated by 1.46, 6.92, and 1.34 times with UV-B radiation, while endogenous H_2O_2 content was also significantly increased. UV-B radiation and exogenous H_2O_2 treatment significantly increased the activities, gene and protein expression of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and isoflavone synthase (IFS) involved in isoflavones synthesis, and there was a synergistic effect with combining treatment. However, these up-regulation effects were suppressed by the supplementary diphenylene iodonium (DPI), which is the inhibitor of NADPH oxidase. Interestingly, the inhibition effect was largely reversed by exogenous H_2O_2, indicating that H_2O_2 was indispensable in regulating the isoflavones synthesis in germinated soybeans under UV-B radiation. Overall, H_2O_2 is an essential signaling molecule, mediating UV-B-induced isoflavone accumulation.

1. Introduction

Germinated soybean is a traditional vegetable food consumed popularly in Asian countries. Numerous researches focused on the breeding and cultivation techniques to improve nutritional value of germinated soybeans (Lee et al., 2007). Notably, UV-B radiation has long been considered as an important regulator for the biosynthesis of secondary metabolites in plants, inducing phenolic compounds, alkaloids, terpenes, carotenoids, and glucosinolates, which are pivotal for the defense systems of plants (Jiao et al., 2015). UV-B radiation is a physical technology without environmental pollution, and has been used for processing vegetables and fruits enriched in valuable phytochemicals. In addition to fresh consumption, the vegetables and fruits with high level of phytochemicals can be used as raw ingredient for functional foods, resulting in the increased ingestion of these health-beneficial substances (Jiao et al., 2015). Isoflavone, a typical group of secondary metabolites, is usually considered as the product of the defense responses of plant to external stimulus (Hahlbrock, Bednarek, Ciolkowski, Hamberger, Heise, Liedgens, & Tan, 2003). Due to their important functions in plant defense system (Du, Huang, & Tang, 2010) and health benefits for human body (Masilamani, Wei, & Sampson, 2012), the metabolism and accumulation of isoflavones were widely studied in the past few years. Our previous studies suggested that UV-B could efficiently promote the accumulation of isoflavones in germinated soybeans (Ma, Wang, Yang, & Gu, 2018); (Ma, Wang, Yang, Zhou, & Gu, 2019); and the endogenous nitric oxide (NO) and inositol 1,4,5-trisphosphate (IP3), have been confirmed as signaling molecules involved in isoflavone accumulation under UV-B (Jiao, Wang, Yang, Tian, & Gu, 2016; Jiao, Yang, Zhou, & Gu, 2016). We also found that UV-B could cause and triggered formation of H_2O_2, which further led to oxidative damage, including cellular damage and lipid peroxidation. It is possible that the production of H_2O_2 under ultraviolet light stress also plays a signal transmission role in the accumulation of isoflavones (Ma et al., 2019).

H2O2 is a direct agent under oxidative stress (Ni et al., 2018); which can respond to various environmental stimuli (Wang, Li, Wang, & Li, 2010). Increasing evidence indicate that H2O2 can act as a local and systemic signal; up-regulating expression of many genes which were activated under environmental stress (Desikan, Hancock, & Neill, 2010). Meanwhile, H2O2 has a long life span, it can cross biological

* Corresponding authors.
E-mail addresses: zhz0731@sina.cn (H. Zhang), yangrq@njau.edu.cn (R. Yang).
[1] Co-first authors.

https://doi.org/10.1016/j.fochx.2022.100331
Received 10 January 2022; Received in revised form 11 April 2022; Accepted 15 May 2022
Available online 18 May 2022

membranes, rapidly diffuse intercellularly, and transfer through the plant cells. As a universal signaling molecule, H2O2 is directly or indirectly linked to the activation of other signaling pathways (Czarnocka & Karpinski, 2018). Notably, H2O2 was also shown to act as a key signaling molecule at the upstream stages of some pathways (Tumova & Tuma, 2011). It was found that H2O2 generated in plants along with NO in response to the pathogen attack and had the same response with NO when mediating defense responses (Neill, Desikan, Clarke, Hurst, & Hancock, 2002). Under UV-B stress, plant growth and metabolism was limited, cell membranes was oxidative damaged, and H_2O_2 was synthesized in large quantities (Ma et al., 2019). Therefore, H_2O_2 also has the potential to be an upstream signaling molecule involved in the isoflavone accumulation under UV-B radiation.

The objective of this study was to investigate whether UV-B could activate the H_2O_2 signaling pathway and then result in the isoflavones accumulation in germinated soybeans; specifically based on the insight into the relevant phytophysiological and biochemical mechanisms. This study could provide a better understanding on synthesis and regulatory mechanism of secondary metabolites such as isoflavones in soybean sprouts, facilitating application in future commercial production such as functional foods.

2. Materials and methods

2.1. Plant materials and germination of soybeans

The soybean cultivar Dongnong was harvested in 2018 and stored at −20 °C until use. For germination, the soybean seeds were soaked in deionized water for 8 h, and then placed in a germinating machine (BX-801, Beixin Hardware Electrical Factory, Zhejiang, China) and germinated for 4 days at 25 °C.

Different treatments were designed as below:

(1) Control: germinated soybeans were cultivated in the dark and sprayed with deionized water every 4 h.
(2) UV-B: germinated soybeans were sprayed with deionized water every 4 h, with UV-B radiation (10 μw/cm^2) for 6 h/day (18 h in dark).
(3) H_2O_2: germinated soybeans were cultivated in the dark and sprayed with 100 μM H_2O_2 aqueous solution every 4 h.
(4) UV-B + H_2O_2: germinated soybeans were sprayed with 100 μM H_2O_2 aqueous solution every 4 h, with UV-B radiation for 6 h/day.
(5) Diphenylene iodonium (DPI): germinated soybeans were cultivated in the dark and sprayed with 20 μM DPI aqueous solution every 4 h. DPI has been claimed to be a specific inhibitor of NADPH oxidases (Davies, Bindschedler, Strickland, & Bolwell, 2006). NADPH oxidases are responsible for the H2O2 generation (Xie, Mao, Zhang, Diwen, Wang, & Shen, 2014). As an inhibitor of the NADPH oxidase, DPI could remove H2O2 production (Davies et al., 2006), inhibits the plant oxidative burst (Delledonne, Xia, Dixon, & Lamb, 1998).
(6) UV-B + DPI: germinated soybeans were sprayed with 20 μM DPI aqueous solution every 4 h, with UV-B radiation for 6 h/day.
(7) DPI + H_2O_2: germinated soybeans were cultivated in the dark and sprayed with both 20 μM DPI and 100 μM H_2O_2 aqueous solutions every 4 h.
(8) UV-B + H_2O_2 + DPI: germinated soybeans were sprayed with 20 μM DPI and 100 μM H_2O_2 aqueous solutions every 4 h, with UV-B radiation for 6 h/day.

2.2. Analysis of H_2O_2 distribution

H_2O_2 distribution in the germinated soybeans was observed using a confocal laser scanning microscope (CLSM, Leica Microsystems, Wetzlar, Germany) with an H_2DCF-DA (2′,7′-dichlorodihydrofluorescein diacetate) fluorescent probe. The soybean cotyledon was sliced to about 100 μm and incubated in 25 μM H_2DCF-DA solution in darkness at 30 °C for 1 h. After washing with phosphate buffer (4 °C), the samples were observed under the CLSM at an excitation and emission wavelength of 488 and 515 nm, respectively (Zhang, Wang, Hu, & Liu, 2015).

2.3. Chemical quantification of endogenous H_2O_2

Chemical quantification of endogenous H_2O_2 was performed according to Li, Xue, Xu, Feng, and An (Li, Xue, Xu, Feng, & An, 2009), which was determined by the formation of a titanium-hydroperoxide complex. Germinated soybeans (10 sprouts) were milled with 50 mL acetone at 4 °C. The mixture was centrifuged (12,000× g, 10 min, 4 °C), followed by adding 20 mL of titanium reagent (20% titanic tetrachloride in concentrated HCl, v/v) and 25 mL of concentrated ammonium solution to form and precipitate titanium-hydroperoxide complex. The mixtures were then centrifuged (10,000× g, 10 min), and the precipitate was dissolved in H_2SO_4 (2 M, 50 mL), followed by centrifugation (10,000× g, 10 min). The final supernatant absorbance was measured at 415 nm.

2.4. Isoflavones analysis

The isoflavones content was determined according to Ma et al. (Ma et al., 2018). The lyophilized sample (0.2 g) was extracted with 6 mL 80% methanol solution at 50 °C for 1 h, centrifuged at 12,000 g for 20 min. The supernatant was filtered with a 0.45 μm micropore filter prior to the high performance liquid chromatography (HPLC) analysis. The HPLC system (Agilent Technologies 1200 series, USA) was equipped with a LC Column (Luna® 5 μm C18(2) 100 A, 250×4.6 mm, Phenomenex, USA). The test parameters were as follows: solvent A, 0.1% acetic acid in water; solvent B, 0.1% acetic acid in acetonitrile; elution gradients, the ratio of solvent A was decreased (87–65%, 50 min), and then increased (65–87%, 1 min); flow rate, 1 mL/min; oven temperature, 35 °C.

2.5. Assay of key enzymes activity related to isoflavones biosynthesis

Ten sprouts of frozen germinated soybeans were homogenized with extraction buffer [50 mM Tris-HCl, pH 8.9, containing 4 mM $MgCl_2$, 15 mM 2-mercaptoethanol, 5 mM ascorbic acid, 1 mM PMSF, 10 μM Leupeptin, 0.15 (w/v) PVP and 10% (v/v) glycerol]. Then the mixture was centrifuged at 13, 000× g, for 20 min (4 °C) and the supernatant was collected to determine the activities of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS), and isoflavone synthase (IFS). The activities of PAL were analyzed using the method described by Han, Li, Jin, Li, Wang, and Zheng (Han et al., 2017). CHS and IFS activities were measured with enzyme-linked immune assay kit (GE, USA) (Jiao et al., 2016).

2.6. Assay of NADPH (nicotinamide adenine dinucleotide phosphate) oxidase activity

NADPH oxidase activities were determined using an A127-1-1 NADPH oxidase assay kit (Nanjing Jiancheng Institute, Jiangsu, China) following the manufacturer's instructions. The protein content of enzyme extracts was determined according to Bradford (Bradford, 1976). An enzyme activity unit is defined as 1 μmol of NADPH per unit time (per minute) was oxidized at 30° C, pH 7.0.

2.7. Gene expression (quantitative real-time PCR, qRT-PCR).

Total RNA was extracted from germinated soybeans using a Takara Plant RNA Kit (Code No. 9769, Takara, China). For the synthesis of first-strand cDNA, certain amount of total RNA was reverse-transcribed using a PrimeScript RT reagent Kit (Code No. DRR037A, Takara, China). The

Table 1
The primers used for QRT-PCR.

Gene	Primer name	Primer sequences
NADPH	Sense	TTGGGGTTTTCTATTGTGGACC
	Anti-sense	GCTTCAACAGATATGTTCCATCAGA
PAL	Sense	CTACCATCACCAATGGGAGCC
	Anti-sense	CTCCCCAGTTTAACGGATCACT
CHS	Sense	GCTTGTTGTCTGTTCTGAG
	Anti-sense	CACCTTCACTGTCTGGAG
IFS1	Sense	GAGAGCTGGCCTCACAGTTC
	Anti-sense	TGCGATGGCAAGACACTACT
IFS2	Sense	TGGAAGTTCGTGAGGAAG
	Anti-sense	ATGGAGATGGTGCTGTTG

sequence-specific primers used in this study for qRT-PCR analysis are listed in Table 1. For each sample, three replications of PCR were performed for real-time quantitative assays using SYBR Premix Ex Taq kit (Code No. RR420A, Takara:) in an ABI sequence detection system (model 7500, Applied Biosystems, CA, USA).

2.8. Western blot analysis

Soybean germinated at the 4th day was harvested for Western blot assays according to [11] described. Tissue lysates were obtained using RIPA buffer containing a protease inhibitor cocktail. After centrifugation, 12 μL of the mixtures containing 40 μg of protein each were loaded into the wall of a 10% (w/v) SDS-PAGE gel and the electrophoresis was performed at 80 V for 2 h. Then the samples were transferred to a 0.45 μm polyvinylidene difluoride (PVDF) membrane (Millipore, USA). Subsequently, the membranes were blocked with 5% nonfat dried milk (Bio-Rad) in Tris-buffer saline with 0.1% Tween 20 (TBST) for 60 min at 25 °C. After that, the membranes were washed with TBST for 5 times, and incubated with primary antibody (anti-PI-PLC, anti-CHS, and anti-IFS) for 10 h at 4 °C, followed by incubation with secondary goat polyclonal antibodies conjugated to horseradish peroxidase (goat anti-rabbit IgG, 1:5000, Bio-Rad; mouse anti-rabbit IgG, 1:5000, Merck Millipore, Germany) for 60 min at 25 °C. Membranes were washed 5 times for 3 min each with TBST. Anti-rubisco antibody was used to

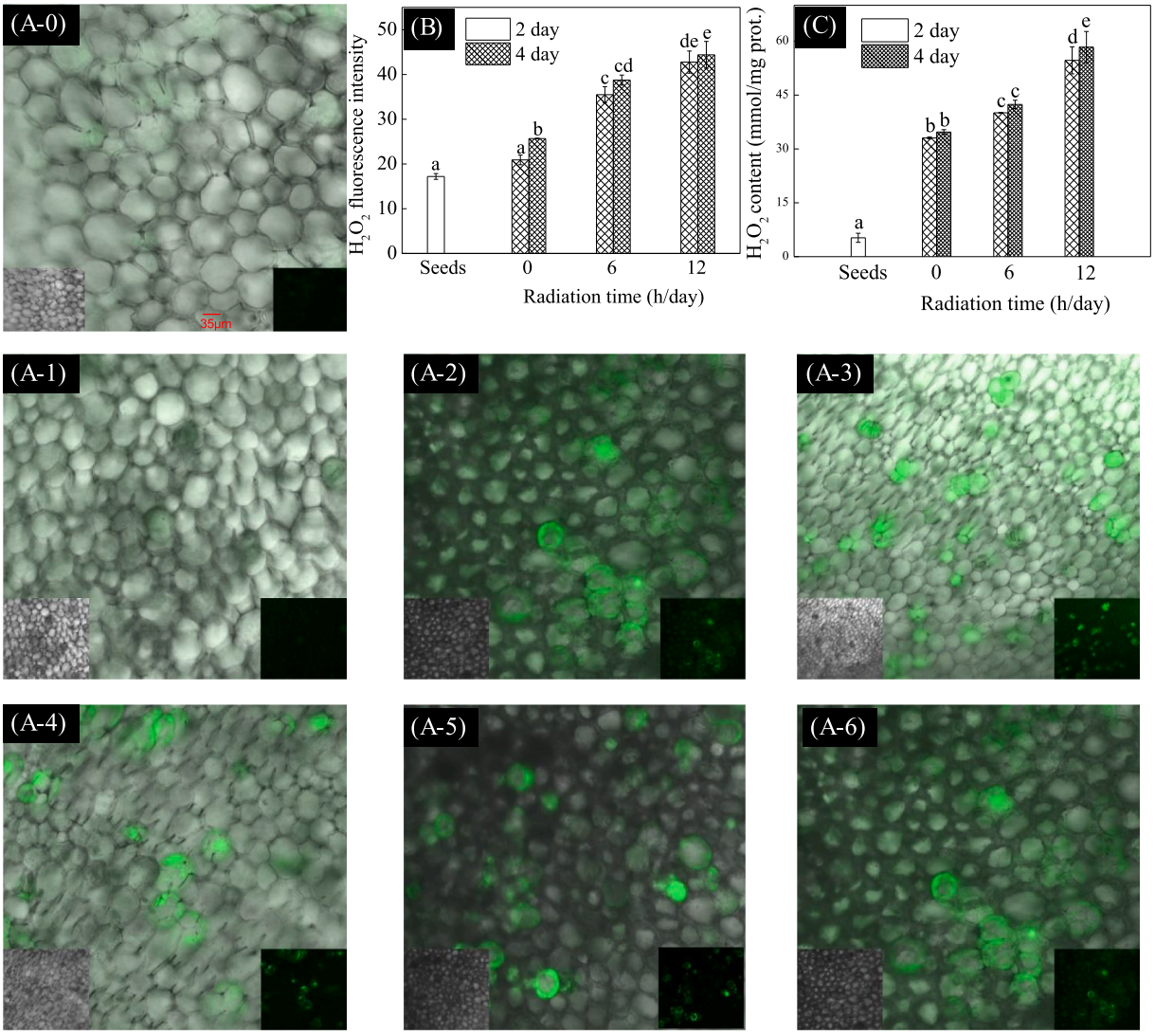


Fig. 1. Staining assays of H_2O_2 production in germinated soybean (A) and relative fluorescence of H_2O_2 (B) and H_2O_2 content (C) of germinated soybean determined using chemical method. A-0, ungerminated soybean seed; A-1, soybean germinated for 2 days; A-2, soybean with UV-B radiation of 6 h/day after germinating for 2 days; A-3, soybean with UV-B radiation of 12 h/day after germinating for 2 days; A-4, soybean germinated for 4 days; A-5, soybean with UV-B radiation of 6 h/day after germinating for 4 days; A-6, soybean with UV-B radiation of 12 h/day after germinating for 4 days. Germinated soybean was stained with H_2DCF-DA and observed with a CLSM at 488 nm excitation and 525 nm emission. Bar = 35 μm. Data are means of three replicates and their standard errors. Different letters above the column indicate significant differences, the same below. The inserted pictures on the CLSM images are bright field (left bottom) and fluorescence channel (right bottom) respectively.

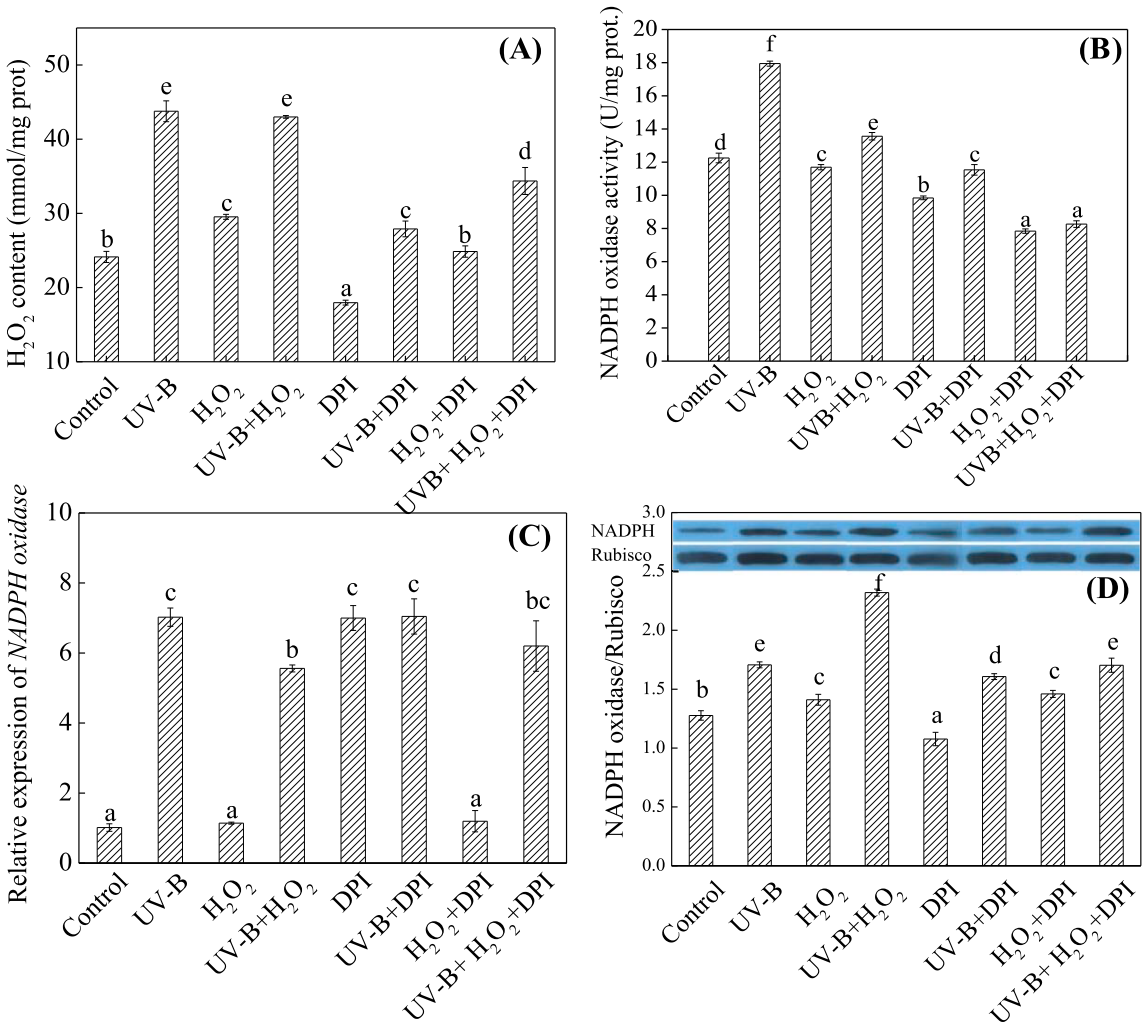


Fig. 2. Effects of UV-B on H_2O_2 production (A), activity (B), gene expression (C) and protein expression (D) of NADPH oxidase in germinated soybean. (D) Histograms represent relative protein levels of germinated soybeans normalized to the corresponding rubisco; the inserted pictures show representative bands.

normalize. SuperSignal® West Dura Extended Duration ECL Substrate (Bio-Rad, Warsaw, Poland) was added to determine the immunocomplexes per corresponding protocol, which were then visualized with an X-ray film system. BandScan 5.0 software was applied to quantify the relative levels of immunoreactivity.

2.9. Statistical analysis

The data were expressed as the means of at least three replications. SPSS 20.0 (SPSS Inc., Chicago, USA) for windows was used to analyze the statistical significance based on ANOVA. The probability value of $p < 0.05$ was considered as statistically significant by using Duncan's test. The figures were created using Origin 8.5 Professional (OriginLab, Northampton, MA, USA).

3. Results

3.1. UV-B induced endogenous H_2O_2 synthesis in germinated soybeans

H_2O_2 content and distribution in germinated soybeans was shown in Fig. 1A. In the absence of UV-B radiation, no obvious fluorescence was observed in soybean seeds and 2-day germinated sprouts (Fig. 1 A-0, A-1), while a slight increase was detected after 4 days of germination (Fig. 1 A-4). Interestingly, a remarkable increase occurred during UV-B radiation exposure. The average H_2O_2 fluorescence intensity of germinated soybean with different radiation time was calculated and summarized (Fig. 1B). Compared with the control (0 h/day), the H_2O_2 fluorescence intensity increased by 51% and 73% with the UV-B radiation of 6 and 12 h/day after germination for 4 days, respectively. To further confirm this result, H_2O_2 accumulation in response to UV-B treatment was monitored by chemical method·H_2O_2 content in germinated soybeans with UV-B radiation was dramatically enhanced, whereas no noticeable changes were detectable for non-radiated samples (Fig. 1C). These results indicated the positive effects of UV-B radiation on H_2O_2 accumulation, which might further mediate the accumulation of isoflavones in germinated soybeans in response to UV-B exposure.

The effects of exogenous H_2O_2 and the NADPH oxidase inhibitor-DPI on the endogenous H_2O_2 content were explored to unravel underlying biomolecular mechanisms. Results showed that UV-B radiation induced an increment of H_2O_2 content by 81% as compared with the control (Fig. 2A). It also significantly up-regulated the activity (Fig. 2B), gene expression levels (Fig. 2C) and protein expression levels (Fig. 2D) of NADPH oxidase. However, the level of UV-B-induced H_2O_2 (UV-B) decreased by 36% when treated with 20 μM DPI (UV-B + DPI). DPI also significantly weakened the enhancement of activity and protein expression of NADPH oxidase in germinated soybeans (DPI and UV-B + DPI). In addition, the application of exogenous H_2O_2 significantly reversed the impact of DPI on the content of endogenous H_2O_2 and the activity (Fig. 2B), gene expression levels (Fig. 2C) and protein expression

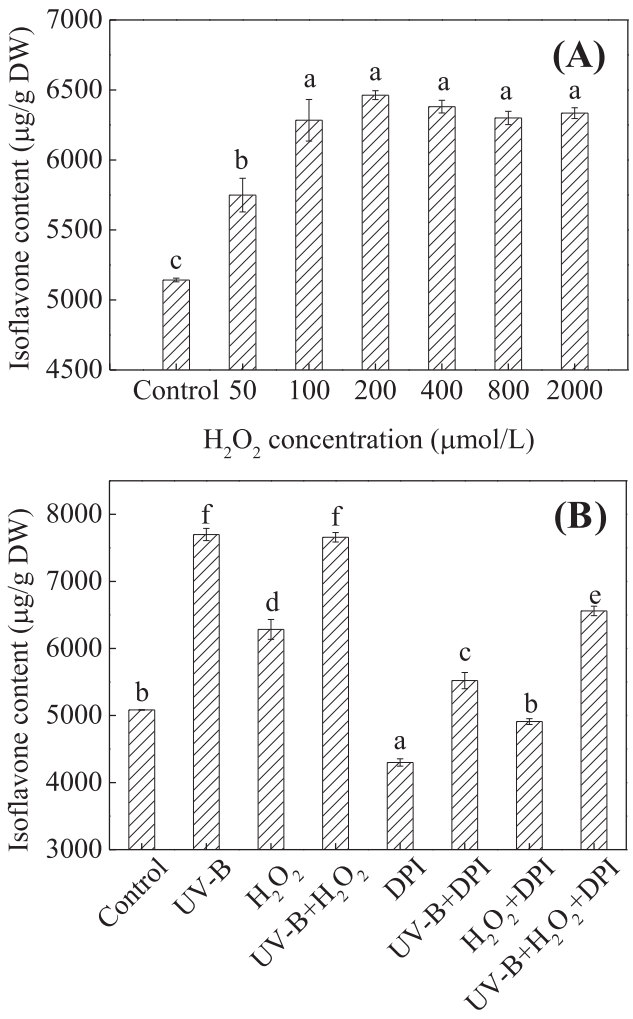


Fig. 3. Effects of H_2O_2 concentration and NADPH oxidase inhibitor on isoflavones content in germinated soybean.

levels (Fig. 2D) of NADPH oxidase (DPI + H_2O_2 and UV-B + H_2O_2 + DPI).

3.2. Effect of exogenous H_2O_2 on isoflavones content in the germinated soybean

As shown in Fig. 3A, isoflavones content increased with the enhanced exogenous H_2O_2 concentration from 5142 μg/g (control) to 6283 μg/g (100 μM H_2O_2 treatment). When the concentration increased up to 100 μM, no further increase in isoflavones content was detected. These showed that H_2O_2 might influence the isoflavones content, and there was a concentration-dependent effect between isoflavones content and H_2O_2 content. The isoflavones content of the germinated soybeans treated with UV-B were much higher than that of the control (Fig. 3B), and DPI abolished isoflavone production under UV-B radiation, while the inhibition of DPI could be reversed by exogenous H_2O_2. Compared with the control, the application of exogenous H_2O_2 used alone could significantly up-regulate isoflavones content. Thus, the data suggested that H_2O_2 was involved in UV-B-induced isoflavone production, which suggest that H_2O_2 is an essential signal for mediating UV-B radiation-activated isoflavone synthesis. UV-B does not directly participate in plant growth and development; instead, it activates its corresponding effectors such as H_2O_2. Subsequently, H_2O_2 can facilitate transducing the external UV-B stress signal to a series of downstream defense reactions (Jiao et al., 2016).

3.3. Effect of UV-B triggered H_2O_2 on activity and expression of key enzymes in germinated soybeans

To further investigate whether UV-B-triggered H_2O_2 was involved in isoflavones accumulation, the effect of exogenous H_2O_2 and DPI on the activity, gene and protein expression of enzymes involved in isoflavones biosynthesis was evaluated under UV-B treatment. Results showed that UV-B radiation significantly promoted the elevation of activity, gene and protein expression level of PAL, CHS, and IFS in germinated soybeans (Fig. 4). The application of exogenous H_2O_2 used alone could also have a similar effect. Compared with the control, the application of DPI decreased the isoflavones content, reduced the activity and protein expression of PAL, and decreased the gene expression of *IFS1*. More noteworthy, DPI significantly weakened the positive effects of UV-B stress on the activity, gene and protein expression of PAL, CHS, and IFS (Fig. 4). Exogenous H_2O_2 could significantly reverse the above decrease induced by DPI in activities, gene and protein expression of the critical enzymes, except for the activity and gene expression of IFS.

4. Discussion

This study investigated the underlying relationships between endogenous H_2O_2 signal transduction pathway and isoflavones accumulation induced by UV-B radiation in germinated soybeans. The accumulation of H_2O_2 (Fig. 1) should be due to the enhancement of NADPH oxidase activity, gene expression and protein expression (Fig. 2B–D). Hideg, Jansen, and Strid (Hideg, Jansen, & Strid, 2013) reported that both low and high doses of UV-B could alter reactive oxygen species (ROS) metabolism including the increase of H_2O_2 content. In addition, Zhang, Chen, Zhang, Li, Li, and Ma (Zhang et al., 2014) found that solar ultraviolet radiation regulated anthocyanin synthesis in apple peel by modulating the generation of ROS via plasma membrane NADPH oxidase. Compared with the control, supplementation of exogenous H_2O_2 also significantly enhanced the endogenous H_2O_2 accumulation (Fig. 2A). These results illustrated that UV-B induced the accumulation of H_2O_2 might play the key role in phenolics synthesis in plant.

Exogenous application of H_2O_2 significantly enhanced the isoflavones content, which were 1.24 times higher than the control (Fig. 3). Wu, Su, Zhang, Liu, Cui, and Liang (Wu et al., 2016) also found that exogenous H_2O_2 addition significantly increased the concentration of anthocyanin. Moreover, UV-B induced the generation of endogenous H_2O_2 (Figs. 1 and 2), Indicating that the UV-B-induced H_2O_2 accumulation might be the pre-event of isoflavones production. Kataria, Jajoo, and Guruprasad (Kataria, Jajoo, & Guruprasad, 2014) revealed that UV-B could affect photosynthetic processes through the generation of ROS. PAL, CHS, IFS are the three key enzymes participating in isoflavone biosynthesis. Li, Ou-Lee, Raba, Amundson, and Last (Li, Ou-Lee, Raba, Amundson, & Last, 1993) suggested that elimination of CHS in *Arabidopsis* could result in UV-hypersensitive phenotypes. Moreover, an *Arabidopsis* mutant with the tolerance of extremely high-dose of UV-B radiation was found to contain constitutively higher levels of phenolic compounds including flavonoids, and have higher expression of *CHS* (Bieza & Lois, 2001). Our previous studies also confirmed that the activity of PAL and IFS were enhanced under UV-B radiation. In the present study; it was revealed that UV-B-triggered H_2O_2 generation led to isoflavones accumulation by up-regulating the activity, gene and protein expression of these key enzymes (Fig. 4, UV-B treatment). Therefore, it was deduced that H_2O_2 could transduce the UV-B signal into downstream defense responses, rapidly induce the transcripts encoding the key enzymes including CHS which is the first enzyme of the branch specific for flavonoids and isoflavonoid biosynthesis (Delledonne et al., 1998); then induced isoflavones accumulation. Compared with UV-B treatment, these up-regulating effects were largely inhibited by adding a specific H_2O_2-scavenger-DPI (Fig. 4, UV-B + DPI treatment). The results also showed that DPI not only suppressed the generation of H_2O_2,

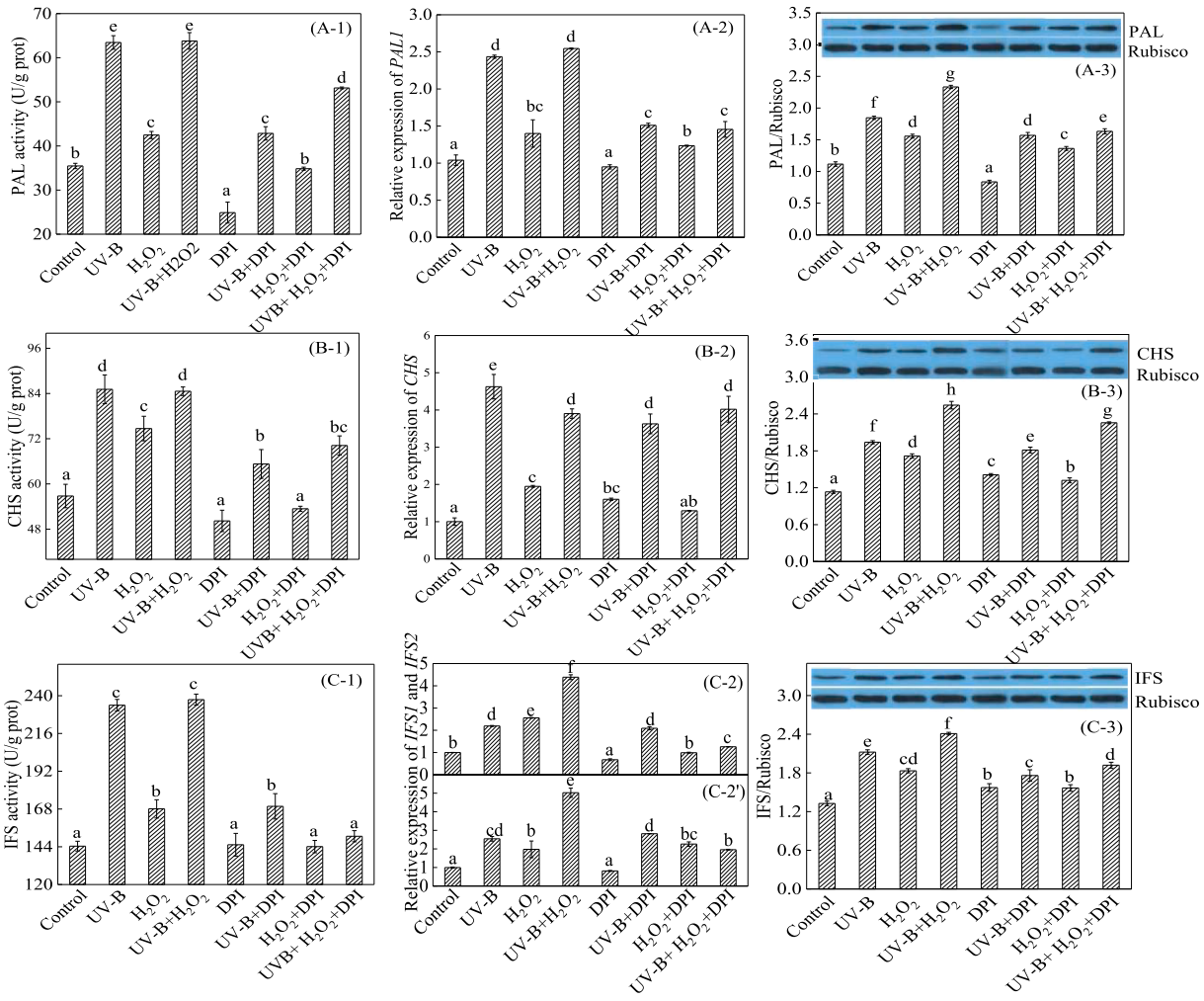


Fig. 4. Effects of UV-B triggered H_2O_2 generation on the activity (1), gene expression (2) and protein expression (3) of PAL (A), CHS (B) and IFS (C) participating in isoflavones synthesis of germinated soybeans. (3) Histograms represent relative protein levels of germinated soybeans normalized to the corresponding rubisco. The inserted pictures show representative bands.

but also significantly inhibited the isoflavones production (Fig. 3B, UV-B + DPI treatment) induced by UV-B stress (Fig. 3B, UV-B treatment). However, the inhibition could be reversed by the addition of exogenous H_2O_2 (UV-B + H_2O_2 + DPI treatment). It might due to that the application of exogenous H_2O_2 increased the endogenous H_2O_2 level, which was similar with the effect of UV-B radiation. Then exogenous-induced endogenous H_2O_2 production activated the key enzymes and accumulation of isoflavones.

5. Conclusion

In conclusion, H_2O_2 triggered by UV-B, induced isoflavone accumulation by regulating the activity, gene and protein expression of enzymes that participate in isoflavone synthesis. DPI abolished both the UV-B-triggered H_2O_2 generation and the UV-B-induced isoflavones production, inhibited the activity, gene and protein expression of enzymes involved in H_2O_2 and isoflavones biosynthesis, while the inhibition of DPI could be reversed by exogenous H_2O_2. In addition, the application of H_2O_2 significantly up-regulated protein expression of CHS and IFS which were the key enzymes related to isoflavones biosynthesis. This study indicated the role of H_2O_2 signaling pathway in mediating isoflavones accumulation under UV-B radiation in germinated soybeans. The process of isoflavone synthesis under UV-B radiation may have complex and multiple signal transduction mechanisms. In the future, it is necessary to further explore the signaling molecule involved in the downstream stages of H_2O_2 pathway, and provide a better understanding on the signaling network mechanism of isoflavone accumulation under UV-B radiation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Funding

This project was funded by Natural Science Foundation of China (32001614, 31801550), Advanced Talents Foundation of QAU (No. 1120038), China Scholarship Council (No. 201806850075), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD) and the Open Project Program of China-Canada Joint Lab of Food Nutrition and Health, Beijing Technology and Business University (No. KFKT-ZJ-2106).

References

Bieza, K., & Lois, R. (2001). An Arabidopsis Mutant Tolerant to Lethal Ultraviolet-B Levels Shows Constitutively Elevated Accumulation of Flavonoids and Other Phenolics. *Plant Physiology, 126*(3), 1105–1115. https://doi.org/10.1104/pp.126.3.1105

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem, 72* (s 1–2), 248–254. https://doi.org/10.1006/abio.1976.9999

Czarnocka, W., & Karpinski, S. (2018). Friend or foe? Reactive oxygen species production, scavenging and signaling in plant response to environmental stresses. *Free Radic Biol Med, 122*, 4–20. https://doi.org/10.1016/j.freeradbiomed.2018.01.011

Davies, D. R., Bindschedler, L. V., Strickland, T. S., & Bolwell, G. P. (2006). Production of reactive oxygen species in Arabidopsis thaliana cell suspension cultures in response to an elicitor from Fusarium oxysporum: Implications for basal resistance. *J Exp Bot, 57*(8), 1817–1827. https://doi.org/10.1093/jxb/erj216

Delledonne, M., Xia, Y., Dixon, R. A., & Lamb, C. (1998). Nitric oxide functions as a signal in plant disease resistance. *Nature, 394*(6693), 585–588. https://doi.org/10.1038/29087

Desikan, R. S. A. H.-M., Hancock, J. T., & Neill, S. J. (2001). Regulation of the Arabidopsis transcriptome by oxidative stress. *Plant Physiol, 127*(1), 159–172. https://doi.org/10.1104/pp.127.1.159

Du, H., Huang, Y., & Tang, Y. (2010). Genetic and metabolic engineering of isoflavonoid biosynthesis. *Appl Microbiol Biotechnol, 86*(5), 1293–1312. https://doi.org/10.1007/s00253-010-2512-8

Hahlbrock, K., Bednarek, P., Ciolkowski, I., Hamberger, B., Heise, A., Liedgens, H., . . . Tan, J. (2003). Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. *Proceedings of the National Academy of Sciences of the United States of America, 100 Suppl 2*(suppl 2), 14569-14576. 10.1073/pnas.0831246100.

Han, C., Li, J., Jin, P., Li, X., Wang, L., & Zheng, Y. (2017). The effect of temperature on phenolic content in wounded carrots. *Food Chemistry, 215*, 116–123. https://doi.org/10.1016/j.foodchem.2016.07.172

Hideg, E., Jansen, M. A., & Strid, A. (2013). UV-B exposure, ROS, and stress: Inseparable companions or loosely linked associates? *Trends Plant Sci, 18*(2), 107–115. https://doi.org/10.1016/j.tplants.2012.09.003

Jiao, J., Gai, Q. Y., Wang, W., Luo, M., Gu, C. B., Fu, Y. J., & Ma, W. (2015). Ultraviolet Radiation-Elicited Enhancement of Isoflavonoid Accumulation, Biosynthetic Gene Expression, and Antioxidant Activity in Astragalus membranaceus Hairy Root Cultures. *J Agric Food Chem, 63*(37), 8216–8224. https://doi.org/10.1021/acs.jafc.5b03138

Jiao, C., Wang, P., Yang, R., Tian, L., & Gu, Z. (2016). IP3 Mediates Nitric Oxide-Guanosine 3',5'-Cyclic Monophosphate (NO-cGMP)-Induced Isoflavone Accumulation in Soybean Sprouts under UV-B Radiation. *J Agric Food Chem, 64*(44), 8282–8288. https://doi.org/10.1021/acs.jafc.6b02633

Jiao, C., Yang, R., Zhou, Y., & Gu, Z. (2016). Nitric oxide mediates isoflavone accumulation and the antioxidant system enhancement in soybean sprouts. *Food Chemistry, 204*, 373–380. https://doi.org/10.1016/j.foodchem.2016.02.147

Kataria, S., Jajoo, A., & Guruprasad, K. N. (2014). Impact of increasing Ultraviolet-B (UV-B) radiation on photosynthetic processes. *J Photochem Photobiol B, 137*, 55–66. https://doi.org/10.1016/j.jphotobiol.2014.02.004

Lee, S. J., Ahn, J. K., Khanh, T. D., Chun, S. C., Kim, S. L., Ro, H. M., … Chung, I. M. (2007). Comparison of isoflavone concentrations in soybean (Glycine max (L.) Merrill) sprouts grown under two different light conditions. *J Agric Food Chem, 55* (23), 9415–9421. https://doi.org/10.1021/jf071861v

Li, J., Ou-Lee, T. M., Raba, R., Amundson, R. G., & Last, R. L. (1993). Arabidopsis Flavonoid Mutants Are Hypersensitive to UV-B Irradiation. *Plant Cell, 5*(2), 171–179. https://doi.org/10.1105/tpc.5.2.171

Li, S. W., Xue, L. G., Xu, S. J., Feng, H. Y., & An, L. Z. (2009). Hydrogen peroxide acts as a signal molecule in the adventitious root formation of mung bean seedlings. *Environmental and Experimental Botany, 65*(1), 63–71. https://doi.org/10.1016/j.envexpbot.2008.06.004

Ma, M., Wang, P., Yang, R., & Gu, Z. (2018). Effects of UV-B radiation on the isoflavone accumulation and physiological-biochemical changes of soybean during germination: Physiological-biochemical change of germinated soybean induced by UV-B. *Food Chemistry, 250*, 259–267. https://doi.org/10.1016/j.foodchem.2018.01.051

Ma, M., Wang, P., Yang, R., Zhou, T., & Gu, Z. (2019). UV-B mediates isoflavone accumulation and oxidative-antioxidant system responses in germinating soybean. *Food Chemistry, 275*, 628–636. https://doi.org/10.1016/j.foodchem.2018.09.158

Masilamani, M., Wei, J., & Sampson, H. A. (2012). Regulation of the immune response by soybean isoflavones. *Immunol Res, 54*(1–3), 95–110. https://doi.org/10.1007/s12026-012-8331-5

Neill, S. J., Desikan, R., Clarke, A., Hurst, R. D., & Hancock, J. T. (2002). Hydrogen peroxide and nitric oxide as signalling molecules in plants. *Journal of Experimental Botany, 53*(372), 1237–1247. https://doi.org/10.1093/jexbot/53.372.1237

Ni, J., Wang, Q., Shah, F. A., Liu, W., Wang, D., Huang, S., … Wu, L. (2018). Exogenous Melatonin Confers Cadmium Tolerance by Counterbalancing the Hydrogen Peroxide Homeostasis in Wheat Seedlings. *Molecules, 23*(4), 799. https://doi.org/10.3390/molecules23040799

Tumova, L., & Tuma, J. (2011). The effect of UV light on isoflavonoid production in Genista tinctoria culture in vitro. *Acta Physiologiae Plantarum, 33*(2), 635–640. https://doi.org/10.1007/s11738-010-0566-y

Wang, Y., Li, J. L., Wang, J. Z., & Li, Z. K. (2010). Exogenous H2O2 improves the chilling tolerance of manilagrass and mascarenegrass by activating the antioxidative system. *Plant Growth Regulation, 61*(2), 195–204. https://doi.org/10.1007/s10725-010-9470-0

Wu, Q., Su, N., Zhang, X., Liu, Y., Cui, J., & Liang, Y. (2016). Hydrogen peroxide, nitric oxide and UV RESISTANCE LOCUS8 interact to mediate UV-B-induced anthocyanin biosynthesis in radish sprouts. *Sci Rep, 6*, 29164. https://doi.org/10.1038/srep29164

Xie, Y., Mao, Y., Zhang, W., Diwen, L., Wang, Q., & Shen, W. (2014). *Reactive Oxygen Species-Dependent Nitric Oxide Production Contributes to Hydrogen-Promoted Stomatal Closure in Arabidopsis*.

Zhang, J., Chen, C., Zhang, D., Li, H., Li, P., & Ma, F. (2014). Reactive oxygen species produced via plasma membrane NADPH oxidase regulate anthocyanin synthesis in apple peel. *Planta, 240*(5), 1023–1035. https://doi.org/10.1007/s00425-014-2120-4

Zhang, D., Wang, H., Hu, Y., & Liu, Y. (2015). Chitosan Controls Postharvest Decay on Cherry Tomato Fruit Possibly via the Mitogen-Activated Protein Kinase Signaling Pathway. *J Agric Food Chem, 63*(33), 7399–7404. https://doi.org/10.1021/acs.jafc.5b01566