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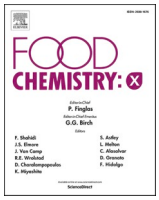

Hydrogen peroxide regulates the biosynthesis of phenolic compounds and antioxidant quality enhancement in lettuce under low nitrogen condition

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ABSTRACT

Reduced nitrogen availability is an efficient strategy for increasing the accumulation of phenolic compounds in vegetables, but related mechanisms remain unknown. Here, the production of hydrogen peroxide (H_2O_2) and its potential roles in regulating phenolic biosynthesis and enhancing the antioxidant quality of lettuce under low nitrogen (LN) conditions were investigated. The LN treatment caused a rapid production of H_2O_2, which effectively increased lettuce quality by enhancing the levels of phenolic compounds and other nutrients such as ascorbic acid, glutathione, soluble sugar, and soluble protein. The increased phenolic content was related to the higher expression levels of phenolic biosynthesis genes, including PAL, CHS, DFR, F35H, and UFGT, and higher photosynthetic capacity after H_2O_2 addition under LN conditions. However, these positive effects were suppressed by dimethylthiourea (DMTU), a scavenger of H_2O_2. These results suggest that H_2O_2 as an important signal molecular mediates the LN-caused phenolic accumulation and antioxidant quality enhancement in lettuce.

1. Introduction

Lettuce (*Lactuca sativa* L.), a leafy vegetable of the family Asteraceae (Compositae), is one of the most widely cultivated vegetables because of its high economic value in the world (Kim, Moon, Tou, Mou, & Waterland, 2016). According to FAOSTAT (2020), the world production of lettuce is approximately 27.7 million tons, with a yield of 14.3 million tons, or 52 % of the worldwide yield in China. According to epidemiological studies, the consumption of fresh vegetables, including lettuce, has positive effects on cancer, cardiovascular diseases, and other chronic diseases (Blekkenhorst et al., 2018; Wallace et al., 2020; Zhou et al., 2019). These positive effects to human health are mainly due to the presence of multiple antioxidant components, such as phenolic compounds, ascorbic acid, glutathione, and carotenoids, in lettuce (Deng, Lin, Xu, Gao, Xie, & Li, 2013; Kim et al., 2016; Souza et al., 2022). Therefore, it is important to explore effective strategies to enhance the biosynthesis of antioxidant components in lettuce to ensure human health.

Phenolic compounds, one of the most widely distributed secondary metabolites in plants, have given increasing attention owing to their powerful antioxidant capacity (Rashmi & Negi, 2020; Shen et al., 2022). Based on their diversity of chemical structures, phenolic compounds can be characterized as phenolic acids, flavonoids, anthocyanins, and stilbene; thereby, being described as more than 8000 natural phenolic compounds identified in plants (Buer, Imin, & Djordjevic, 2010). Phenolic compounds are biosynthesized by various enzymes of the phenylpropanoid pathway, in which phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol-4 reductase (DFR), and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) are key enzymes (Lillo, Lea, & Ruoff, 2008; Shen et al., 2022). The metabolism of phenolic compounds in plants is regulated by various environmental factors, of which nitrogen availability is one of the most important ones (Souza et al., 2022; Sytar et al., 2018; Zhou et al., 2018). It has been well reported that reduced nitrogen supply generally results in the shift from N-based to C-based compounds, thereby promoting the accumulation of C-based metabolites (mainly including the phenolic compounds in plants) (Le Bot, Benard, Robin, Bourgaud, & Adamowicz, 2009). For example, transcriptome and metabolome analyses have revealed that nitrogen mediates phenolic biosynthesis by coordinating the activation of specific genes or by regulating carbon and nitrogen metabolism (Zheng et al., 2021). The phenolic content in lettuce was negatively correlated with nitrogen supply levels, showing that every type of phenolic compound decreased with increasing N supply (Qadir, Siervo, Seal, & Brandt,

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2017). Similarly, our previous studies showed that lettuce cultivated under low nitrogen levels had higher enzyme activities and transcriptional levels of genes involved in phenolic biosynthesis, exhibiting higher phenolic content (Zhou et al., 2018; Zhou et al., 2019). Interestingly, optimizing nitrogen supply also benefits in high nitrogen use efficiency and low nitrate accumulation in vegetables, thereby potentially making positive effects on human health and the environment (Qadir et al., 2017). However, the regulatory signals involved in nitrogen levels that mediate the biosynthesis of phenolic compounds in lettuce are poorly understood.

Reactive oxygen species (ROS), particularly H_2O_2, play a central role in plant responses to various environmental stresses (Asgher et al., 2021; Considine & Foyer, 2021). Reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is the main enzyme for the H_2O_2 production, which has been recognized as a signal compound at low concentrations in regulating plant growth and development (Considine & Foyer, 2021). Various studies have established the positive roles of exogenous H_2O_2 in stimulating the accumulation of phenolic compounds in plants (Khandaker, Boyce, & Osman, 2012; Leon-Lopez et al., 2020; Swieca, 2015). In lentil sprouts, the higher total phenolic content, PAL activity, and antioxidant capacity were observed after elicitation with H_2O_2 (Swieca, 2015). In *Arabidopsis*, exogenous H_2O_2 activates the transcription of several phenolic biosynthesis genes, leading to the biosynthesis of flavonoids and anthocyanins (Shi, Liu, Wei, & Chan, 2018). In addition, higher accumulation of ROS has been widely reported in plants, such as *Arabidopsis*, *Hypericum perforatum*, and maize under nitrogen deficiency (Kovacik, Dresler, Peterkova, & Babula, 2020; Shin, Berg, & Schachtman, 2005; Wang et al., 2021). Interestingly, rapid production of H_2O_2 within a few hours in plants has been observed after nitrogen deficiency (Shin et al., 2005). The biosynthesis of many secondary metabolites, including phenolic compounds, is widely believed to be the result of plant responses to stress (Buer et al., 2010; Sytar et al., 2018). Therefore, H_2O_2 may play a potential role in regulating phenolic biosynthesis in plants under low nitrogen availability; however, the underlying mechanism is unknown.

In the present study, reduced nitrogen supply was applied to systematically analyze the response characteristics of phenolic compounds and endogenous H_2O_2 content in lettuce. Subsequently, the effects of H_2O_2 and its scavenger, dimethylthiourea (DMTU), on the growth characteristics, the profile of phenolic compounds, and transcript levels of genes involved in phenolic biosynthesis and antioxidant capacity were investigated. The results of this study may reveal the regulatory role of H_2O_2 in mediating the biosynthesis of phenolic compounds in lettuce under low nitrogen conditions.

2. Materials and methods

2.1. Plant materials and experimental design

Lettuce (Ziluoma) seeds were sterilized with 80 % (v/v) ethanol for 10 min, washed three times with distilled water, and then soaked in distilled water for 12 h. The soaked seeds were placed on a nylon net floating on 0.5 mmol/L $CaCl_2$ solution and germinated in an artificial climate chamber, where the light intensity was 300 μmol m^{-2} s^{-1}, photoperiods were 16/8h (day/night), the temperature was 25/16℃ (day/night), and relative humidity was 60 %. After 8 days, the uniform seedlings were transplanted into nutrient solution, which contained 2 mM nitrate as the nitrogen source: 1.0 mmol/L $Ca(NO_3)_2$, 2.0 mmol/L K_2SO_4, 0.74 mmol/L KH_2PO_4, and 1.0 mmol/L $MgSO_4$ (micronutrients were provided by Hoagland solution). After 12 days, the plants were transferred to plastic containers filled with 1 L of 0.05 mmol/L nitrate solution as the treatment of low nitrogen supply (LN), which contained 0.025 mmol/L $Ca(NO_3)_2$, 2.0 mmol/L K_2SO_4, 0.74 mmol/L KH_2PO_4, 1.0 mmol/L $MgSO_4$, and 0.975 mmol/L $CaCl_2$. Control plants were still cultured in 2 mmol/L nitrate solution (CK). For the H_2O_2 treatment, lettuce plants were sprayed once with about 5 mL of 10 mmol/L H_2O_2 per plant before the treatments. For the DMTU (a scavenger of H_2O_2) treatment, 0.3 mmol/L DMTU was added to the nutrient solution. Each experiment was repeated at least thrice. After 3 days, the plant height was measured using a rule, and the number of leaves were recorded. Then, the fresh weight of shoot and root were determined using an electronic balance. The fresh leaf samples were all collected and stored at −80 ℃ until the completion of following assays.

2.2. Determination of chlorophyll content and photosynthetic parameters

The fresh lettuce leaves were mixed with 80 % (v/v) acetone for the extraction of chlorophyll, and the contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids were measured according to the method of Arnon (1949). Photosynthetic parameters, such as the net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and water use efficiency (WUE), were measured using a photosynthesis system (CIRAS-3, Hansatech ltd., King's Lynn, UK).

2.3. Determination of hydrogen peroxide and histochemical detection

The H_2O_2 content in fresh leaves was measured following the method described by Junglee, Urban, Sallanon, and Lopez-Lauri (2014). The samples were ground into homogenates with 1.5 mL of 0.1 % (v/v) trichloroacetic acid (TCA). After centrifugation, the extracts were then mixed with 1 mM potassium iodide in 50 mM phosphate buffer (pH 7.0). The absorbance was recorded at 390 nm, and the H_2O_2 content was determined using a standard curve plotted with H_2O_2.

Histochemical detection of H_2O_2 was performed using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) following the method described by Liu, Huang, Lin, and Sun (2020). Briefly, leaves were immersed in 10 μM DCFH-DA in 50 mM phosphate buffer (pH 6.0) by vacuum infiltration for 10 min in the dark. The stained leaves were washed with phosphate buffer and observed under a fluorescence microscope (Axioscope A1, Carl Zeiss, Germany).

2.4. Determination of phenolic compounds

Phenolic compounds were extracted according to the method described by Zhou et al. (2018). Fresh lettuce leaves were ground into homogenates with 1.5 mL 80 % methanol solution (*v/v*). After centrifugation (12,000×*g* at 4 ℃) for 10 min, the extracts were filtered through 0.45 μm PTFE filters and used for the analysis of phenolic compounds.

Total phenolic content was quantified using the Folin-Ciocalteu method, and the results were determined as gallic acid equivalents (GAE) in mg g^{-1} of fresh weight (FW) (Deng et al., 2013). Total flavonoid content was determined according to the method described by Jia, Tang, and Wu (1999), and the results were expressed as catechin equivalents (CE) in mg g^{-1} FW. The anthocyanin content was determined according to the pH differential method, and the results were calculated as cyanidin 3-glucoside equivalents (CGE) in μg g^{-1} FW (Kaulmann, Jonville, Schneider, Hoffmann, & Bohn, 2014).

Individual phenolic compounds in the extracts were analyzed using an Agilent 1200 high-performance liquid chromatography system (Agilent Technologies, Santa Clara, CA, USA) equipped with a mass spectrometer (MS) (Zhou et al., 2018). Chromatographic separation was performed using a C18 column (250 × 4.6 nm, 5 μm) with water/methanol gradient. Mobile phase A was 0.1 % formic acid in water and mobile phase B was 100 % methanol. The flow rate was 41.0 mL min^{-1} and the content of phenolic acids and flavonoids or anthocyanins were detected at 280 nm or 520 nm, respectively. Mass spectrometry was carried out using an electrospray source in the negative ion mode with a full scan range of 100 to 1200 *m/z*. The phenolic acid content was calculated according to a standard sample of chlorogenic acid, and the flavonoid and anthocyanin contents were calculated according to a standard sample of rutin. The results were expressed as μg g^{-1} FW.

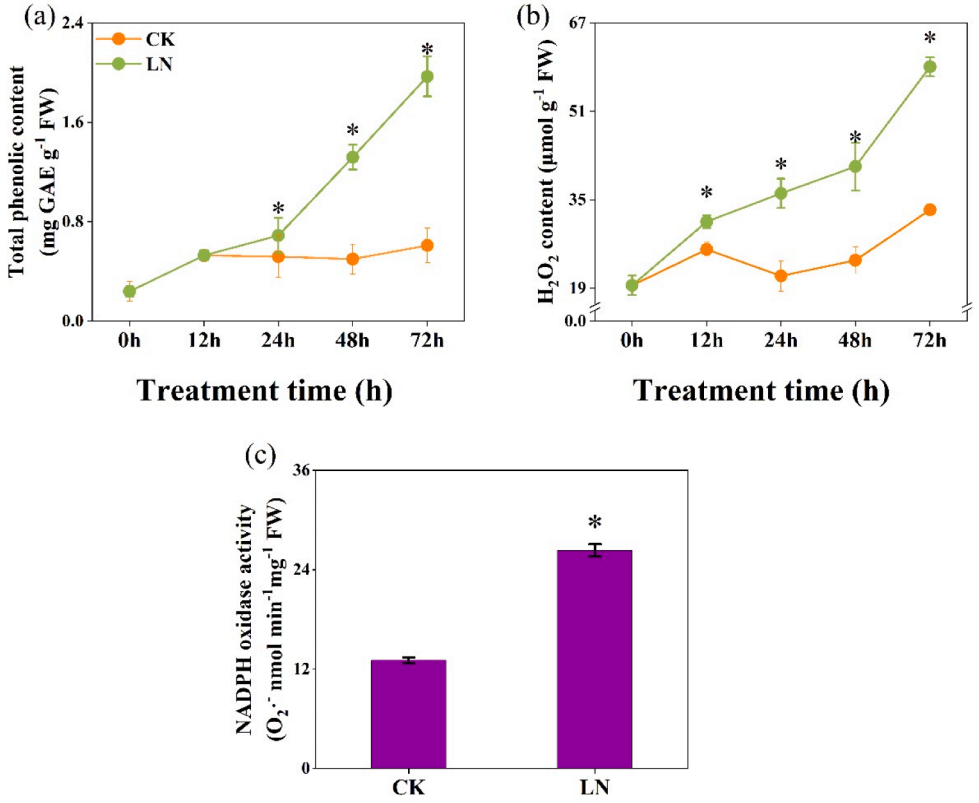

Fig. 1. Changes of total phenolic content (a), H_2O_2 content (b), and NADPH oxidase activity (c) in lettuce cultivated either in adequate (CK) or low nitrogen (LN) solution. The total phenolic and H_2O_2 contents were determined after 0, 12, 24, 48, and 72 h of the treatment. The NADPH oxidase activity was determined after 72 h of the treatment. * indicates significant difference between CK and LN at $P < 0.05$.

2.5. Determination of NADPH oxidase activity

The NADPH oxidase activity was determined following the methods of Sun, Liu, Zhou, Lu, Jin, & Lin (2017). NADPH oxidation was calculated by measuring absorbance at 470 nm (NADPH, $\varepsilon = 21.6\ mM^{-1}\ cm^{-1}$).

2.6. RNA extraction and gene expression analysis

Total RNA was extracted from fresh leaves using a plant RNA purification kit (Sangon Biotech Co., ltd., Shanghai, China) and subsequently reverse-transcribed into cDNA using HiScript II QRT SuperMix (Vazyme Biotech Co., ltd., Nanjing, China). Quantitative real-time PCR analyses were carried out using ChamQ™ SYBR Color qPCR Master Mix (Vazyme Biotech Co., ltd., Nanjing, China), and the gene expression was determined by the $2^{-\Delta\Delta CT}$ method (Zhou, Zheng, Lv, Wang, Liang, & Li, 2022).

2.7. Determination of ascorbic acid, glutathione, soluble sugar, and soluble protein

The ascorbic acid (AsA) was extracted from leaf samples with 5 % (*v/v*) metaphosphoric acid and measured using the reductive methods described by Zhang & Kirkham (1996). The glutathione (GSH) was extracted from leaf samples with 3 % (v/v) trichloroacetic acid containing 5 mM ethylenediaminetetraacetic acid and determined fluorometrically following the methods described by Owens and Belcher (1965). The soluble sugar was extracted with 80 % (*v/v*) ethanol, and then the supernatant was mixed with 5 % (*v/v*) phenol dissolved in 98 % (*v/v*) sulfuric acid and incubated for 1 h. The absorbance was recorded at 485 nm (Dubois, Gilles, Hamilton, Rebers, & Smith, 1951). Soluble protein content was measured following the method described by Bradford (1976).

2.8. Determination of antioxidant activities

The antioxidant capacity of lettuce was reflected by the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging capacity and ferric reducing antioxidant power (FRAP), which were measured according to the methods described by Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, and Hawkins Byrne (2006). The extraction of fresh samples was performed using the same method as the determination of phenolic compounds. For DPPH scavenging capacity analysis, after incubation at 37°C for 30 min, the mixtures of the extracts and DPPH solution were recorded at 515 nm. The DPPH scavenging capacity was determined following the formula: DPPH scavenging activity (%) = 1 − (absorbance of sample/absorbance of control) × 100 %. For FRAP analysis, the extracts and FRAP reagent were mixed for 10 min. After measuring the absorbance at 593 nm, the results were calculated based on the calibration curve of the Fe^{2+} solution and were expressed as μM $Fe^{2+}\ g^{-1}$ FW.

2.9. Statistical analysis

The results are expressed as the mean ± standard deviation (SD) of at least three repetitions. All data were analyzed by one-way analysis of variance (ANOVA) using SPSS 23.0 (SPSS Inc., Chicago, USA), followed by the least significant difference (LSD) test at $P < 0.05$.

3. Results

3.1. Effects of low nitrogen treatment on total phenolic, H_2O_2, and NADPH oxidase activity

The total phenolic content was relatively constant over 72 h under CK treatment, whereas an obviously enhancement in total phenolic

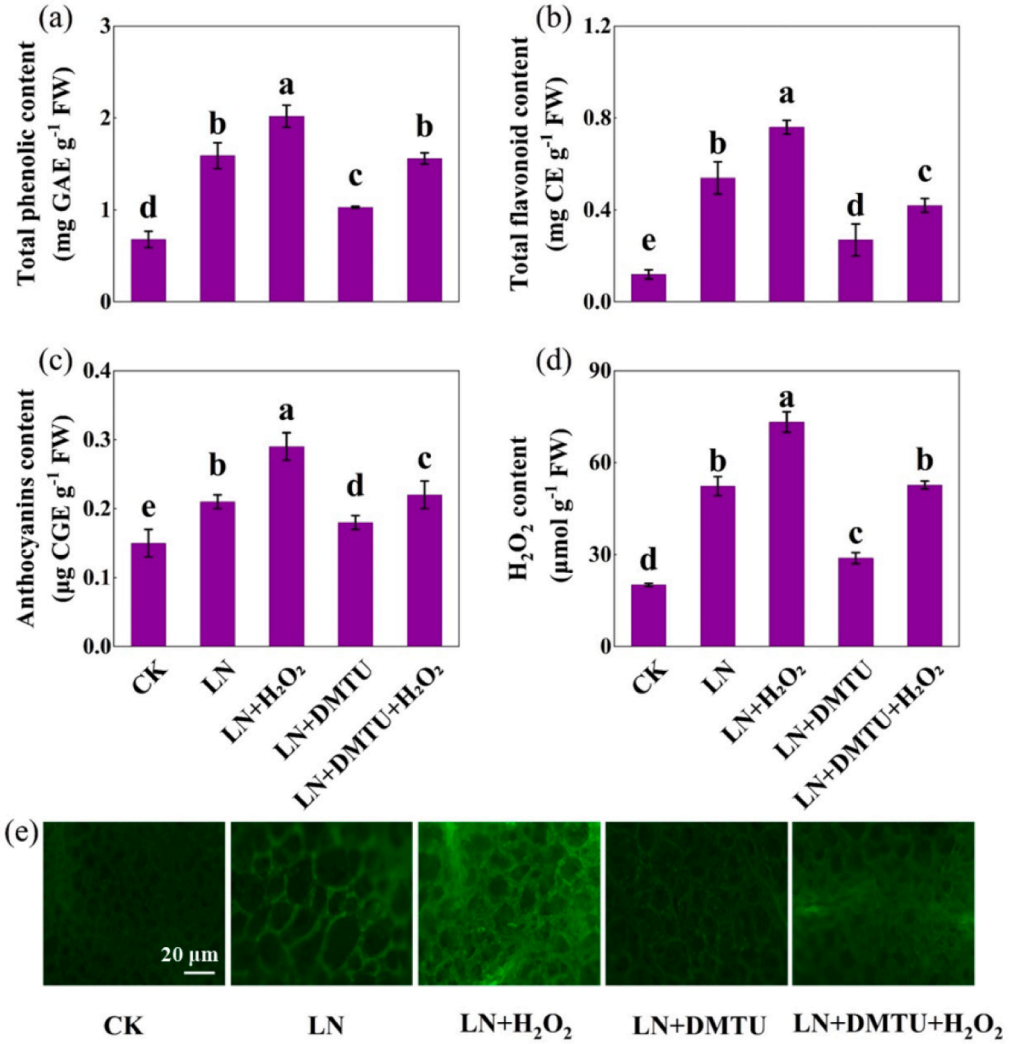

Fig. 2. Effects of H_2O_2 and DMTU on the contents of total phenolic (a), flavonoid (b), anthocyanins (c), and H_2O_2 (d) in lettuce cultivated either in adequate (CK) or low nitrogen (LN) solution. Histochemical visualization of H_2O_2 was performed with DCFH-DA staining (e). The lettuce samples were collected at 72 h after treatment. Different letters indicate a significant difference at $P < 0.05$.

content was found after 24 h in LN-treated lettuce and was approximately 4.4-fold higher than that in the CK-treated lettuce (Fig. 1a). Under LN treatment, endogenous H_2O_2 transiently increased after 12 h and then showed a gradual increase, reaching a maximum of 2.1-fold increase after 72 h of LN treatment (Fig. 1b). As shown in Fig. 1c, LN-treated lettuce showed higher NADPH oxidase activity, which might be attributed to higher H_2O_2 accumulation under LN treatment.

3.2. *Effects of H_2O_2 and DMTU on phenolic compounds*

Compared with the CK treatment, the increased accumulation of phenolic compounds was observed in LN-treated lettuce. As shown in Fig. 2a-c, LN treatment increased the total phenolic, flavonoid, and anthocyanin contents by 135.5 %, 347.2 %, and 42.2 %, respectively, which further increased by 26.8 %, 41.0 %, and 34.4 %, respectively, in lettuce leaves treated with exogenous H_2O_2. However, DMTU treatment prominently decreased the levels of total phenolics, flavonoids, and anthocyanins in LN-treated lettuce, while this inhibition was reversed by simultaneous treatment with H_2O_2. HPLC analysis identified eleven individual phenolic compounds in lettuce, and higher content of the most individual phenolic compounds were shown in LN-treated lettuce leaves (Table 1). Except for chlorogenic acid, the most of the identified phenolic compounds were further increased by exogenous H_2O_2, and similar inhibitory effects were observed after DMTU treatment. To further confirm whether LN-induced phenolic accumulation was triggered by endogenous H_2O_2 production, the H_2O_2 content in lettuce was determined by chemical analysis (Fig. 2d) and histochemical staining (Fig. 2e), which showed the same pattern as phenolic accumulation.

3.3. *Effects of H_2O_2 and DMTU on gene expressions of enzymes involved in phenolic biosynthesis*

In plants, the biosynthesis of phenolic compounds is catalyzed by some key enzymes, such as PAL, CHS, F3H, DFR, and UFGT. LN treatment caused an enhancement in the relative gene expression of PAL, CHS, F3H, DFR, and UFGT, and exogenous H_2O_2 further increased these by 13.2 %, 54.0 %, 32.4 %, 34.2 %, and 27.9 %, respectively, in lettuce leaves (Fig. 3). In contrast, DMTU treatment significantly abolished the LN-induced expression improvement of PAL, CHS, F3H, DFR, and UFGT, whereas these effects were partially reversed after simultaneous treatment with H_2O_2. These results suggest that H_2O_2 stimulated phenolic accumulation by regulating the expression of genes involved in phenolic biosynthesis in LN-treated lettuce.

Table 1
Effects of H_2O_2 and DMTU on contents of individual phenolic compound (μg/g FW) identified by HPLC analysis in lettuce cultivated either in adequate (CK) or low nitrogen (LN) solution. The lettuce samples were collected at 72 h after treatment.

Phenolic compound	Treatments				
	CK	LN	LN + H_2O_2	LN + DMTU	LN + DMTU + H_2O_2
Phenolic acids					
Caftaric acid	40.1 ± 5.0 bc	43.8 ± 6.0 b	51.8 ± 8.2 a	26.9 ± 5.7 d	32.2 ± 2.0 cd
Chlorogenic acid 1	58.9 ± 19.0 c	302.9 ± 43.6 ab	337.2 ± 50.9 a	60.1 ± 15.9 c	265.0 ± 30.0 b
Chlorogenic acid 2	18.4 ± 2.3 c	24.3 ± 2.5 b	26.5 ± 1.4 ab	21.0 ± 2.3 c	29.7 ± 3.0 a
Caffeic acid	196.5 ± 33.9 c	654.5 ± 61.2 b	762.8 ± 128.6 a	211.3 ± 40.8 c	623.9 ± 28.4 b
Chicoric acid	28.6 ± 8.9 c	49.5 ± 5.1 b	61.3 ± 7.7 a	36.0 ± 5.0 c	55.0 ± 3.7 ab
Di-*O*-caffeoylquinic acid	48.7 ± 5.7 d	65.6 ± 4.1 c	97.6 ± 13.0 a	54.8 ± 4.1 d	79.3 ± 5.3 b
Flavonoids					
Quercetin-3-*O*-malonylglucoside	59.5 ± 6.9 d	136.9 ± 16.6 b	181.1 ± 27.7 a	53.0 ± 21.4 d	90.1 ± 6.1 c
Quercetin derivatives 1	85.4 ± 20.5 c	216.2 ± 29.4 b	338.0 ± 81.5 a	97.0 ± 10.6 c	200.6 ± 19.5 b
Quercetin derivatives 2	46.0 ± 6.5 c	132.0 ± 14.4 b	199.7 ± 43.7 a	62.8 ± 5.5 c	127.1 ± 13.9 b
Apigenin conjugate	50.5 ± 5.5 d	139.5 ± 13.5 b	169.1 ± 26.6 a	70.4 ± 8.3 c	126.9 ± 5.2 b
Anthocyanin					
Cyanin-3-(6′′-*O*-acetyl)-glucoside	31.9 ± 0.9 d	98.8 ± 12.0 b	131.8 ± 26.2 a	50.4 ± 7.9 c	85.8 ± 2.7 b

Different letters within the same row indicate significant differences at $P < 0.05$.

3.4. Effects of H_2O_2 and DMTU on ASA, GSH, soluble sugar, soluble protein, and antioxidant capacity

Compared with the CK treatment, the LN-treated lettuce exhibited higher contents of AsA, GSH, soluble sugar, and soluble protein (Fig. 4a-d). Based on the LN treatment, exogenous H_2O_2 further improved the contents of AsA, GSH, soluble sugar, and soluble protein by 29.8 %, 13.5 %, 22.9 %, and 12.7 %, respectively, whereas DMTU treatment decreased the content by 15.0 %, 51.8 %, 25.1 %, and 5.5 %, respectively. Further treatment with H_2O_2 partially reversed the DMTU-induced inhibition on AsA, GSH, soluble sugar, and soluble protein under LN conditions. In addition, the trends in DPPH free radical scavenging capacity and FRAP value appeared similar to the changes in the above antioxidant compounds in lettuce leaves (Fig. 4e and f).

3.5. Effects of H_2O_2 and DMTU on growth characteristics, chlorophyll content, and photosynthetic parameters

Compared with the CK treatment, the LN treatment inhibited plant height, leaf number, and shoot fresh weight by 24.3 %, 13.8 %, and 49.8 %, respectively; however, H_2O_2, DMTU, or their combination had no significant effects on these parameters (Fig. 5a). The chlorophyll *a* content decreased significantly by 13.1 %, but chlorophyll *b*, carotenoids, and total chlorophyll content did not change obviously under the LN treatment, compared with the CK treatment (Fig. 5b). Application of H_2O_2 significantly increased the chlorophyll *a*, chlorophyll *b*, carotenoid, and total chlorophyll contents by 20.7 %, 9.9 %, 25.0 %, and 16.0 %, respectively, under LN treatment. Under the LN treatment, Pn, Gs, Tr, and WUE were significantly reduced, whereas exogenous H_2O_2 treatment markedly improved the levels of Pn, Gs, Tr, and WUE by 12.9 %, 34.3 %, 16.0 %, and 12.2 %, respectively (Fig. 5c). However, DMTU treatment significantly reversed the H_2O_2-caused decreases in Pn, Gs, Tr, and WUE, whereas further addition of H_2O_2 reduced these effects.

4. Discussion

In recent years, plant-derived phenolic compounds, have given considerable attention for their function in inhibiting the risks of cancer, cardiovascular disease, and neurodegenerative diseases (Shen et al., 2022; Wallace et al., 2020). Therefore, effective measures to increase the levels of phenolic compounds in vegetables would be beneficial to human health. Higher accumulation of phenolic compounds after reducing the nitrogen supply has been reported in many species (Kovacik et al., 2020; Qadir et al., 2017; Zhou et al., 2018), but the underlying mechanism involved in this process remains unclear. Our results illustrate the potential role of H_2O_2 in reducing nitrogen supply-caused phenolic accumulation in lettuce. We observed that LN treatment induced a gradual increase in total phenolic content and H_2O_2 production, and their time-course results demonstrated that H_2O_2 might act as a signaling molecule involved in mediating phenolic biosynthesis in lettuce under LN conditions (Fig. 1). The exogenous application of H_2O_2 or an H_2O_2 scavenger (DMTU) further confirmed the involvement of H_2O_2 in regulating phenolic biosynthesis in lettuce under LN conditions. Exogenous H_2O_2 caused a further increase in H_2O_2 production and subsequently increased the phenolic content in LN-treated lettuce leaves, while DMTU treatment reversed these positive effects (Fig. 2 and Table 1). Several studies have also found that exogenous H_2O_2 largely improves the accumulation of phenolic compounds in wax apple, lentil sprouts, and chickpea (Khandaker et al., 2012; Leon-Lopez et al., 2020; Swieca, 2015). These results suggest that the increased accumulation of phenolic compounds in LN-treated lettuce is regulated by H_2O_2 as a potential signaling molecule.

H_2O_2 regulates various metabolic processes in plants under both normal and stressful conditions (Considine & Foyer, 2021). Oxidative bursts and subsequent H_2O_2 accumulation in plant cells are the most common consequences when plants are faced to diverse environmental stresses (Asgher et al., 2021; Liu et al., 2020). An obvious enhancement of H_2O_2 in lettuce was observed after 12 h of LN treatment, with a gradual improvement with prolonged treatment (Fig. 1b), indicating that LN-induced H_2O_2 production was very rapid. A similar rapid accumulation of H_2O_2 within several hours after nitrogen deficiency has also been reported in Arabidopsis (Shin et al., 2005). In plants, plasma membrane-associated NADPH oxidase is the key enzyme responsible for H_2O_2 production (Sun et al., 2017). Our results found that LN-treated lettuce showed significant increase in NADPH oxidase activity (Fig. 1c), suggesting that H_2O_2 accumulation in LN-treated lettuce might be due to the induction of NADPH oxidase. A lower concentration of H_2O_2 helps in signaling, but its higher content induces oxidative damage to biomacromolecules, leading to the inhibition of plant growth (Considine & Foyer, 2021). Plants have developed multiple strategies to reduce oxidative damage caused by ROS under stressful conditions. Phenolic compounds, an important group of secondary metabolites, have strong antioxidant capacities to scavenge ROS, which are widely believed to be responses to stress (Buer et al., 2010; Shi et al., 2018; Sytar et al., 2018). A higher antioxidant activity, as indicated by the DPPH free radical scavenging capacity and FRAP value, was found in the LN-treated lettuce after H_2O_2 treatment (Fig. 4e and f). As mentioned above, the higher levels of phenolic compounds might be a result of lettuce defensive responses to alleviate the oxidative damage caused by low nitrogen levels.

Understanding the profiles and biosynthesis of phenolic compounds will provide better insights into how phenolic metabolism is involved in plant responses to endogenous H_2O_2 under LN conditions. In this study, compared with LN treatment, the levels of total phenolics, flavonoids, anthocyanins, and most individual phenolic compounds identified in lettuce increased obviously under LN + H_2O_2 treatment (Fig. 2a-c and

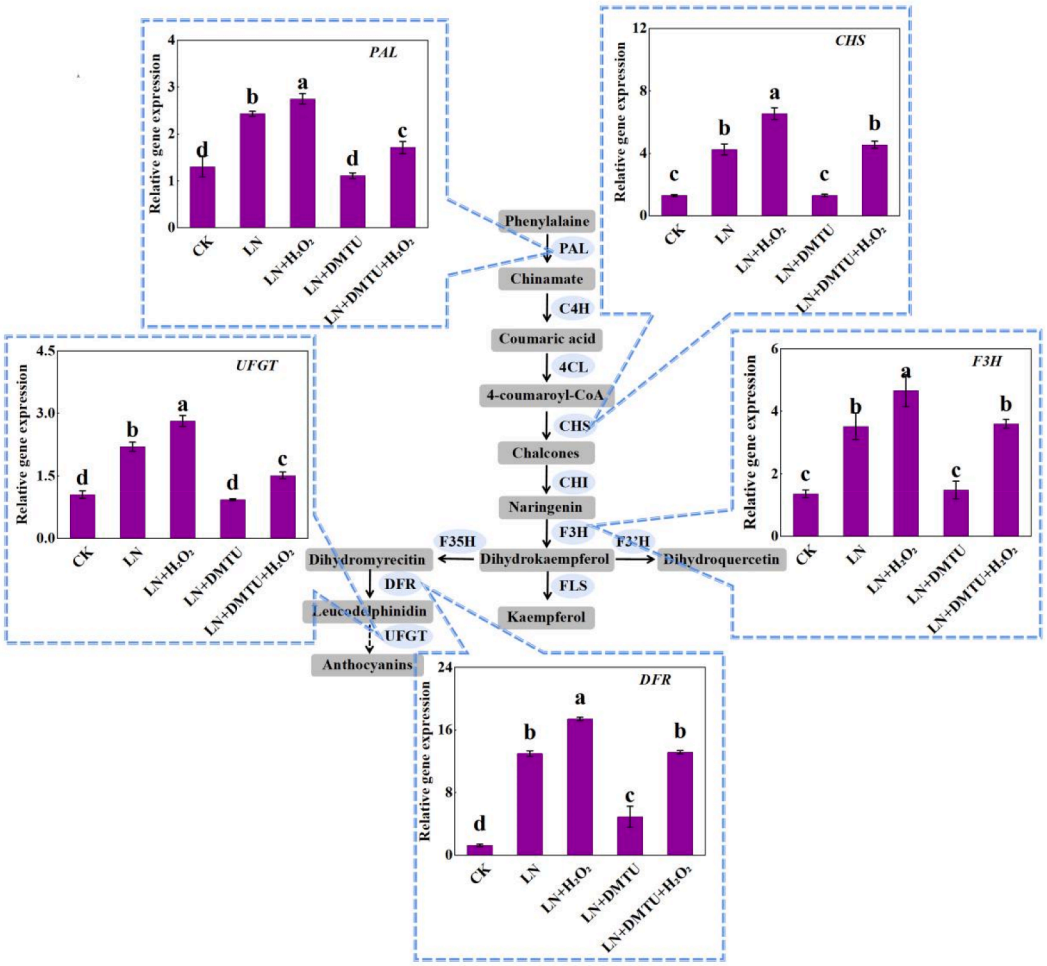

Fig. 3. Effects of H_2O_2 and DMTU on gene expressions of PAL, CHS, F3H, DFR, and UFGT in lettuce cultivated either in adequate (CK) or low nitrogen (LN) solution. The lettuce samples were collected at 72 h after treatment. PAL, phenylalanine ammonia-lyase; CHS, chalcone synthase; F3H, flavanone 3-hydroxylase; DFR, dihydroflavonol-4-reductase; UFGT, UDP-glucose: flavonoid 3-*O*-glucosyltransferase. Enzymes investigated in this study are shown in the simplified scheme for the phenolic biosynthesis pathway. Different letters indicate a significant difference at $P < 0.05$.

Table 1). However, DMTU addition decreased the content of phenolic compounds, indicating that H_2O_2 production was responsible for LN-induced phenolic biosynthesis in lettuce. The phenylpropanoid pathway is the main biosynthesis pathway for phenolic compounds and can be stimulated via various environmental factors (Buer et al., 2010; Sytar et al., 2018). For instance, a global transcript analysis in Arabidopsis showed that most of the transcript levels in the phenylpropanoid pathway are largely upregulated by depleted mineral nutrients, including nitrogen (Lillo et al., 2008). In pears, NADPH oxidase-mediated H_2O_2 plays an essential role in melatonin-induced phenolic accumulation via regulating the expression of genes such as PAL, CHS, F3H, and DFR (Sun et al., 2021). In agreement with previous studies, endogenous H_2O_2 strengthened the de novo biosynthesis of phenolic compounds under LN conditions, as demonstrated by the increased expression of some genes associated with phenolic synthesis, including PAL, CHS, F3H, DFR, and UFGT (Fig. 3). These observations suggested that the accumulation of endogenous H_2O_2 could mediate the phenolic biosynthesis in lettuce at the transcriptional level under LN conditions.

Phenolic compounds are a large group of C-rich metabolites, and their biosynthesis is dependent on the available carbon resources produced by chloroplasts, the main photosynthetic organelles in plants (Shen et al., 2022). Low availability of N results in the inhibition of chlorophyll biosynthesis and photosynthetic capacity because N is essential for chlorophyll structure (Qadir et al., 2017). H_2O_2 has been reported in regulating many physiological processes, such as photorespiration, stomatal movement, photosynthesis, and growth development (Considine & Foyer, 2021; Kato, Miura, Ido, Ifuku, & Sakamoto, 2009). The results obtained from our study showed that LN treatment significantly decreased chlorophyll *a* content, whereas exogenous H_2O_2 significantly increased the contents of chlorophyll *a*, chlorophyll *b*, carotenoids, and total chlorophyll in LN-treated lettuce (Fig. 5b). Likewise, LN treatment also largely decreased gas exchange attributes, such as Pn, Gs, Tr, and WUE (Fig. 5c), indicating that it caused reduced CO_2 fixation and photosynthetic efficiency, ultimately resulting in photoinhibition. However, the application of H_2O_2 effectively alleviated the inhibition of photosynthetic parameters caused by LN treatment (Fig. 5c). Excessive ROS accumulation in chloroplasts is the most vital negative impact of nitrogen deficiency, which hampers photosynthesis by damaging chloroplast components, such as thylakoid membranes, chlorophyll, and D1 protein (Kato et al., 2009). Therefore, we speculated that the increased photosynthetic capacity induced by H_2O_2 may be due to the function of phenolic compounds in scavenging excessive ROS in chloroplasts under low nitrogen conditions. As a result, the lettuce growth, such as shoot fresh weight, plant height, and leaf number, was also inhibited by LN treatment, while application of H_2O_2 had no obvious positive effects on the above growth parameters (Fig. 5a). This discrepancy may be attributed to the three days of short-term treatment that did not cause significant changes in biomass production after H_2O_2 application in our study. Similarly, a previous study found that H_2O_2 treatment increased chlorophyll content,

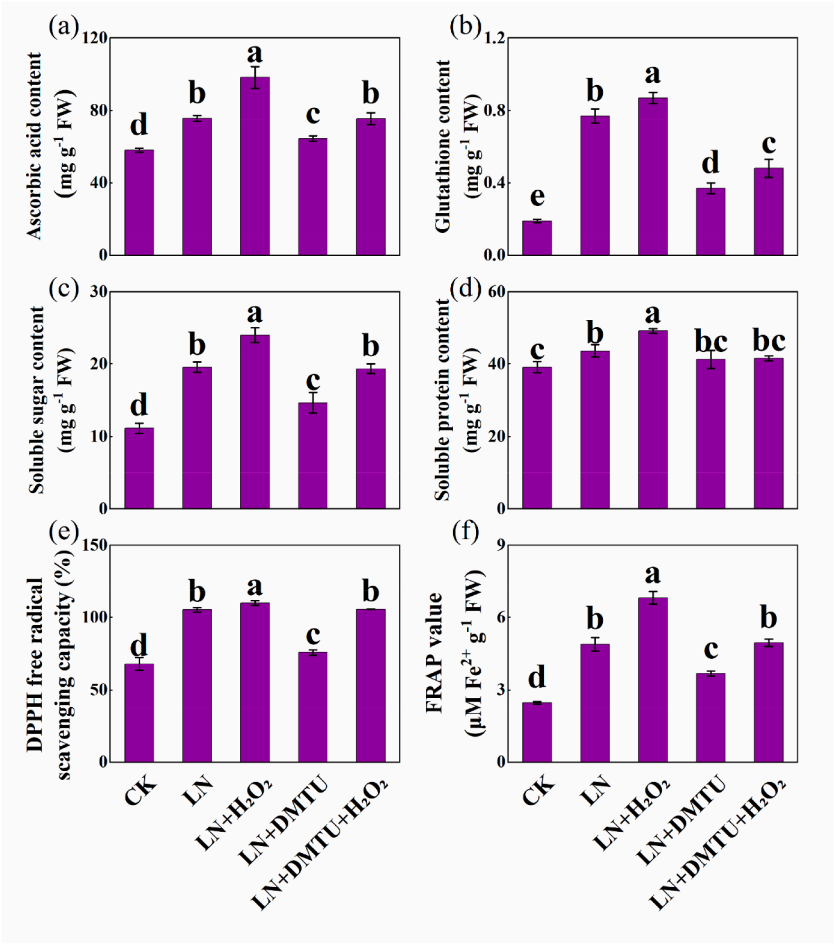

Fig. 4. Effects of H_2O_2 and DMTU on ascorbic acid (a), glutathione (b), soluble sugar (c), soluble protein (d), DPPH free radical scavenging capacity (e), and FRAP value (f) in lettuce cultivated either in adequate (CK) or low nitrogen (LN) solution. The lettuce samples were collected at 72 h after treatment. Different letters indicate a significant difference at $P < 0.05$.

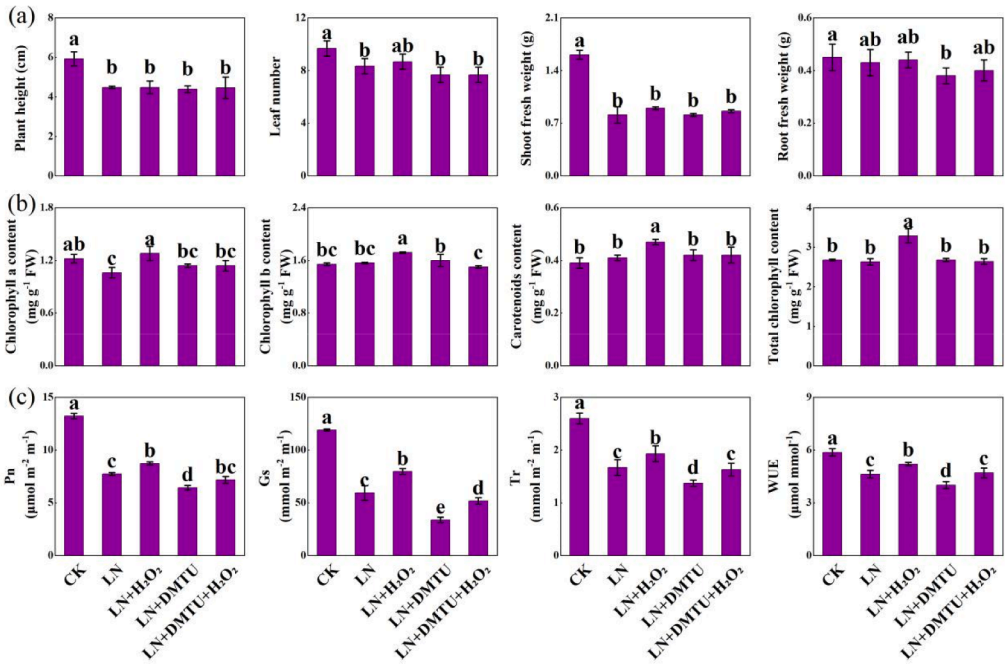

Fig. 5. Effects of H_2O_2 and DMTU on (a) plant height, leaf number, shoot fresh weight, and root fresh weight; (b) chlorophyll *a*, chlorophyll *b*, carotenoids, and total chlorophyll contents; (c) net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and water use efficiency (WUE) in lettuce cultivated either in adequate (CK) or low nitrogen (LN) solution. The lettuce samples were collected at 72 h after treatment. Different letters indicate a significant difference at $P < 0.05$.

photosynthetic capacity, and growth of tomatoes under copper stress (Nazir, Hussain, & Fariduddin, 2019). Thus, H_2O_2 might enhance the supply of carbon resources in LN-treated lettuce by reducing oxidative damage to chloroplasts, thereby increasing the biosynthesis of phenolic compounds.

In lettuce, ASA and GSH are two bioactive compounds with high antioxidant capacities, showing health benefits in reducing the risk of chronic diseases (Blekkenhorst et al., 2018; Kim et al., 2016). The significant enhancement of AsA and GSH content in LN-treated lettuce was caused by H_2O_2 application, but DMTU treatment significantly reversed these positive effects (Fig. 4a and b). Similarly, exogenous H_2O_2 application enhanced ASA and GSH levels in cauliflower, tomato, rice, and wheat under abiotic stress (Asgher et al., 2021; Ellouzi, Oueslati, Hessini, Rabhi, & Abdelly, 2021; Liu et al., 2020; Nazir et al., 2019). Moreover, soluble sugar and soluble protein levels have also been regarded as the important nutritional factors that affect the quality of vegetables (Wallace et al., 2020). As a possible consequence of the reduced oxidative damage to chloroplasts caused by H_2O_2 application (Fig. 5), improved photosynthetic capacity resulted in the accumulation of soluble sugars and proteins (Fig. 4c and d). These results are in line with an earlier study on apples, which indicated that exogenous H_2O_2 remarkably improved the accumulation of soluble sugars and soluble proteins by increasing photosynthesis capacity (Khandaker et al., 2012). Based on these findings, we concluded that the application of H_2O_2 could effectively improve the quality of lettuce under LN conditions by increasing not only the biosynthesis of phenolic compounds but also the accumulation of ASA, GSH, soluble sugars, and proteins.

5. Conclusion

Our results revealed that H_2O_2 as an important signal molecular regulated the biosynthesis of phenolic compounds and antioxidant quality of lettuce under low nitrogen conditions. LN treatment induced the rapid accumulation of H_2O_2, which increased the levels of phenolic compounds through upregulating the genes associated with phenolic biosynthesis. Moreover, this might be partially due to the enhanced photosynthetic capacity after H_2O_2 treatment, which supplies more carbon resources for phenolic biosynthesis. Interestingly, H_2O_2 was also beneficial for the accumulation of ASA, GSH, soluble sugars, and soluble proteins in LN-treated lettuce, which could potentially make lettuce more nutritious. These results identify the relationship between endogenous H_2O_2 production and phenolic accumulation in LN-treated lettuce, providing a promising strategy to improve the production of phenolic compound-rich vegetables with high antioxidant quality.

CRediT authorship contribution statement

Weixuan Wang: Methodology, Data curation, Writing – original draft, Funding acquisition. **Chenglong Zhang:** Methodology, Visualization. **Meixin Shang:** Methodology, Visualization. **Haofeng Lv:** Visualization, Writing – review & editing. **Bin Liang:** Writing – review & editing. **Junliang Li:** Conceptualization, Writing – review & editing. **Weiwei Zhou:** Conceptualization, Methodology, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition.

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.

Data availability

Data will be made available on request.

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