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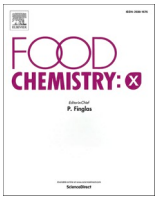

Elicitation with hydrogen peroxide promotes growth, phenolic-enrichment, antioxidant activity and nutritional values of two hydroponic lettuce genotypes

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ABSTRACT

Dietary vegetables rich in bioactive compounds are major responsible for promoting human health. Herein, the effect of hydrogen peroxide (H_2O_2), an important signaling compound, on growth and quality of two hydroponic lettuce genotypes was investigated. The maximum enhancement of growth traits was shown in lettuce elicited with 10 mmol/L H_2O_2, while 40 mmol/L H_2O_2 significantly reduced above growth traits. H_2O_2 elicitation increased pigment contents and photosynthetic process, which consequently caused enhancements of phenolic compounds, ascorbic acid, glutathione, carotenoids, soluble sugars, free amino acids, soluble protein, minerals, and antioxidant capacity, while above alterations appeared in a genotype-dependent manner. The phenolic accumulation was correlated with improved activity of phenylalanine ammonia lyase (PAL) and expression levels of genes related to phenolic biosynthesis, including PAL, chalcone synthase, flavanone 3-hydroxylase, dihydroflavonol-4 reductase, and UDP-glucose: flavonoid 3-*O*-glucosyltransferase. Therefore, elicitation with H_2O_2 is a promising strategy to develop lettuce with high bioactive compounds and biomass.

1. Introduction

Lettuce (*Lacuca sativa* L.) is one of the popularly consumed leaf vegetables in the world (Kim, Moon, Tou, Mou, & Waterland, 2016). According to the Food and Agriculture Organization of the United Nations, a total of about 27.0 million tons of lettuce was produced worldwide, and China held the highest production with more than 53.2% in 2021. Lettuce has good nutritional values, which not only contains low amounts of calories, fat, and sodium, but also has high levels of minerals and a lot of bioactive compounds, such as carotenoids, vitamins, and phenolic compounds (Collado-Gonzalez, Pinero, Otalora, Lopez-Marin, & Del Amor, 2022; Kim et al., 2016). There is documented evidence suggesting that the presence of phenolic compounds is the main responsible for the health beneficial properties of lettuce, such as antioxidant capacity, cardiovascular protective, anti-inflammatory, and anticancer (Rashmi & Negi, 2020; Wang, Li, Ge, & Lin, 2020). Epidemiological studies have also demonstrated the positive correlation between high lettuce consumption and low risks of chronic diseases (Kim et al., 2016; Wang et al., 2020).

Phenolic compounds are an important group of plant-specialized metabolites synthesized by the phenylpropanoid pathway in plants (Daryanavard, Postiglione, Muhlemann, & Muday, 2023). The first and key enzyme in the phenylpropanoid pathway is phenylalanine ammonia lyase (PAL), which is a controlling step in the biosynthesis of phenolic compounds (Lillo, Lea, & Ruoff, 2008). The reduction of phenolic compounds in plants was closely related to the decreased PAL activity. Chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol-4 reductase (DFR), flavonoid 3′,5′-hydroxylase (F35H), and UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) are also key enzymes involving in the biosynthesis of different phenolic compounds, such as phenolic acids, flavonoids, and anthocyanins (Daryanavard et al., 2023; Wang et al., 2022a). The phenolic metabolism is a complex process, and phenolic accumulation in plants is generally caused by various biotic and abiotic stresses (Yang et al., 2023; Zlotek, Swieca, & Jakubczyk, 2014). Recent studies have widely reported the positive effects of stress elicitors, such as methyl jasmonate, melatonin, light, salt, and temperature, on accumulation of secondary metabolites including phenolic compounds in vegetables (Bahcesular, Yildirim, Karaçocuk,

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Kulak, & Karaman, 2020; Collado-Gonzalez et al., 2022; Lim, Park, Kim, Jeong, & Kim, 2012; Wang, Leng, Zhu, Wang, Gu, & Yang, 2022; Zlotek et al., 2014). However, the applied stresses also cause the overproduction of reactive oxygen species (ROS), which in turn results in the plant growth inhibition and yield decline (Lim et al., 2012; Liu, Kang, Zhao, Liu, Zhang, & Zhang, 2019; Yang et al., 2023). In addition, it should be also considered for the potential adverse impacts of applied industrial chemicals on surrounding environment. Therefore, innovative agriculture practices are urgently required to ensure the stable yields, high quality, and health environment in sustainable vegetable production systems.

Stress elicitors lead to oxidative stress by stimulating oxidative bursts and subsequent ROS accumulation (Considine & Foyer, 2021). On the other hand, these ROS, especially hydrogen peroxide (H_2O_2), also act as signaling compounds in regulating cell growth and development, photosynthesis, stomatal movement, and plant stress responses (Asgher, Ahmed, Sehar, Gautam, Gandhi, & Khan, 2021; Considine & Foyer, 2021; Liu, Huang, Lin, & Sun, 2020). Various studies have established the involvement of H_2O_2 in stimulating the phenylpropanoid pathway to improve the biosynthesis of phenolic compounds as plant resistance to environmental changes (Liu et al., 2019; Wang et al., 2022a). In addition, the elicitation of H_2O_2 has also illuminated as an effective technique to increase the production of bioactive compounds including phenolic compounds in vegetables (Liu et al., 2019; Sun et al., 2021). In lentil sprouts, elicitation of H_2O_2 has been found to enhance phenolic content by increasing tyrosine and phenylalanine ammonia-lyases activities (Swieca, 2015). In chickpea sprouts, the H_2O_2 soaking treatment enhanced the phenolic content and antioxidant activity while reduced the content of phytic acid (Leon-Lopez et al., 2020). The application of H_2O_2 enhanced leaf photosynthesis, fruit growth and yield, phenolic content, and antioxidant properties in wax apple (*Syzygium samarangense*) (Khandaker, Boyce, & Osman, 2012). Importantly, H_2O_2 is a promising clean elicitor that is easily broken down by plant or soil enzymes and does not cause environment contamination. However, the roles of H_2O_2 elicitation in regulating plant growth, phenolic biosynthesis, and other nutritional values of lettuce are unknown.

In this study, the effects of H_2O_2 on growth, bioactive compounds mainly including phenolic compounds, mineral profiles, and antioxidant properties of lettuce were systematically studied. Furthermore, the photosynthetic characteristics and gene expression profiles related to phenolic biosynthesis were also evaluated to explore mechanisms of H_2O_2-induced changes in lettuce growth and quality. We presume that H_2O_2 elicitation can be applied as an innovative method to increase the nutritional properties while maintain the yield potential of lettuce, as well as lead to the practical implications for agricultural production of functional vegetables.

2. Materials and methods

2.1. Plant materials and treatment

The experiment was conducted using two genotypes of green (Lvluo) and red (Ziluoma) leaf lettuce, which were purchased from Beijing Golden Land Agricultural Technology Research Institute (Beijing, China). The even-sized seeds were surface sterilized by 80% (v/v) ethanol for about 10 min and rinsed thoroughly using deionized water. After germination in the dark, uniform seedings were transplanted to plastic containers filled with nutrient solution: 2.0 mmol/L $Ca(NO_3)_2$, 4.0 mmol/L KNO_3, 0.74 mmol/L KH_2PO_4, and 1.0 mmol/L $MgSO_4$. The micronutrients were provided following the Hoagland solution. The growth conditions were as follows: 16 h/25 °C day and 8 h/16 °C night cycle, light intensity of 300 μmol m^{-2} s^{-1}, and relative humidity of 60%. After 15 days, the plants were sprayed once daily with five different concentrations of H_2O_2: 0 mmol/L (control treatment, CK), 5 mmol/L (H5), 10 mmol/L (H10), 20 mmol/L (H20), and 40 mmol/L (H40). The experiment was carried out in a completely randomized design with five replicates. After 3 days, the growth parameters were measured. The fresh leaves were sampled and stored at −20 °C for further biochemical analyses.

2.2. Leaf pigments and photosynthetic parameters analysis

Fresh leaves (0.1 g) were ground with 80% (v/v) acetone. Then, the supernatant was used to determine the concentrations of chlorophyll (Chl) a, Chl b, and carotenoids by a spectrophotometer (UV-1601, Rayleigh Ltd., China.) after centrifuged at 12 000 g for 15 min at 4 °C (Arnon, 1949). For the analysis of photosynthetic parameters, net photosynthetic rate (Pn), stomatal conductance (Gs), transpiration rate (Tr), and water use efficiency (WUE) of the fully expanded leaves were determined by a portable photosynthesis system (CIRAS-3, Hansatech Ltd., King's Lynn, UK) from 9 to 11 am.

2.3. Analysis of phenolic compounds

The extraction of phenolic compounds was conducted by homogenizing 0.1 g lettuce samples with 1.5 mL 80% methanol solution (v/v) (Zhou et al., 2018). After centrifugation at 12 000 g for 10 min at 4 °C, the supernatant filtered with 0.45 μm PTFE filter was used to the analysis of phenolic contents. The total phenolic content was measured according to the Folin-phenol method and calculated as gallic acid equivalents (GAE) in mg g^{-1} fresh weight (FW) (Zlotek et al., 2014). The total flavonoids content was measured following the aluminum chloride colorimetric method with slight modifications and expressed as catechin equivalents (CE) in mg g^{-1} FW (Jia, Tang, & Wu, 1999). The modified pH differential method was used to measure the anthocyanin content and calculated as cyanidin 3-glucoside equivalents (CGE) in mg g^{-1} FW (Kaulmann, Jonville, Schneider, Hoffmann, & Bohn, 2014).

Phenolic profiles were analyzed by high-performance liquid chromatography (HPLC) based on the methods of Zhou et al. (2018). The Agilent 1200 HPLC system (Agilent, CA, USA) coupled with a diode array detection (DAD) and C18 column (5 μm, 250 × 4.6 mm). The mobile phase A (0.1% formic acid solution) and B (100% methanol) were used as gradient solvent systems, respectively. The elution procedure was as follows: 0–1 min, 5% B; 1–5 min, 5%–30% B; 5–10 min, 30%–50% B; 10–12 min, 50% B; 12–16 min, 50%–90% B; 16–18 min, 90%–95% B; 18–20 min, 95% B; and 20–22 min, 5% B. The flow rate and injection volumes were 4.0 mL min^{-1} and 3 μL, respectively. The column temperature was maintained at 35 °C. The phenolic acids and flavonoids or anthocyanins were measured at 280 nm or 520 nm, respectively. Standards of chlorogenic acid or rutin was used to quantify the contents of phenolic acids or flavonoids and anthocyanins, respectively.

2.4. Gene expression and PAL activity analysis

Frozen samples (50 mg) were ground with liquid nitrogen, and the total RNA was extracted using the plant RNA purification kit purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The first cDNA synthesis was carried out in a 10 μL volumes following the manufacturer's protocol of HiScript II QRT SuperMix (Vazyme Biotech Co., Ltd., Nanjing, China). Then, the qRT-PCR analysis was performed by ChamQ™-SYBR Color qPCR Master Mix (Vazyme Biotech Co., Ltd., Nanjing, China) using an ABI QuantStudio 3 real-time PCR system (Applied Biosystems, Foster City, CA, USA). The total reaction volume was 20 μL, which contained 10 μM primer, 10 μL ChamQ SYBR Green I, and 1 μL diluted cDNA. Thermocycling was carried out under following conditions: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The sequence-specific primers of enzymes (PAL, CHS, F3H, DFR, F35H, and UFGT) in phenolic biosynthesis were presented in Table S1 following the previous studies (Kitazaki et al., 2018; Wang et al., 2022a). The relative expression levels were determined using the $2^{-\Delta\Delta CT}$ method with *60S rRNA* as the internal reference.

The PAL activity was calculated using ultraviolet colorimetric

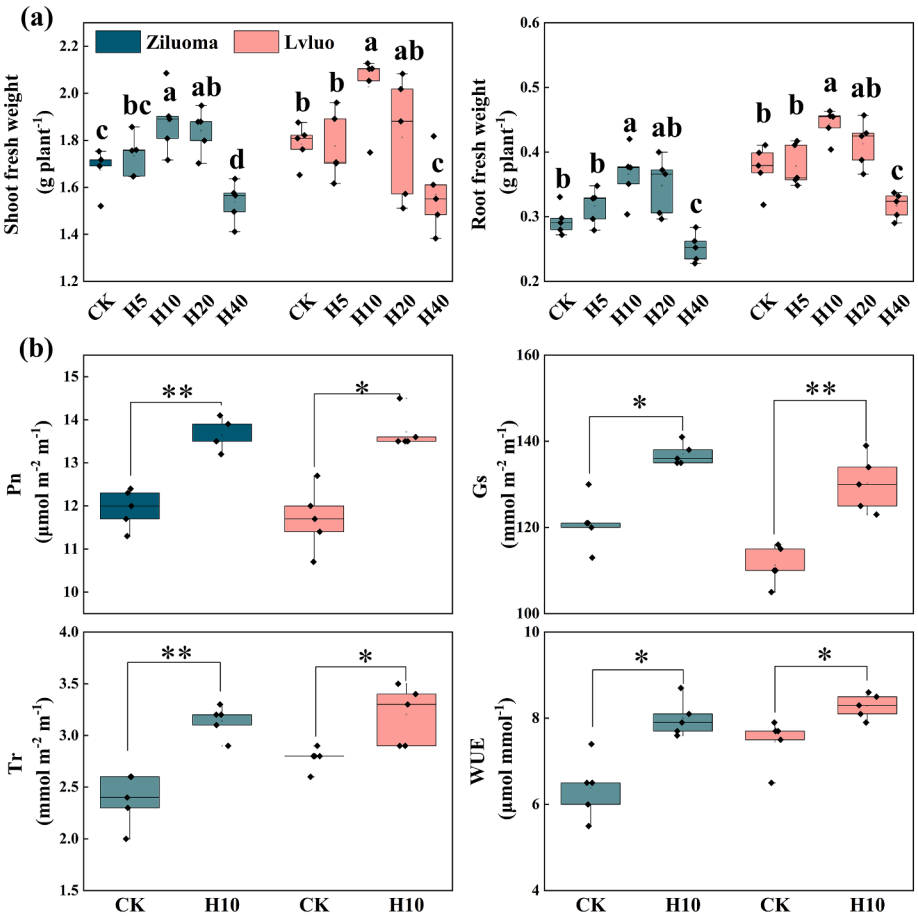

Fig. 1. Effect of H_2O_2 on biomass (a) and photosynthetic parameters (b) of two lettuce genotypes. Different letters indicate significant difference at $P < 0.05$. * and ** indicate significant differences between treatment with $P < 0.05$ and $P < 0.01$, respectively. Pn, net photosynthetic rate; Gs, stomatal conductance; Tr, transpiration rate; WUE, water use efficiency.

method following the PAL activity assay kit purchased from Sangon Biotech Co., Ltd. (Shanghai, China). One unit of PAL activity was expressed as U mg^{-1} protein, where one U was defined as 0. 1 change at 290 nm per min.

2.5. Ascorbic acid and reduced glutathione analysis

The ascorbic acid (AsA) content was assayed according to the method of Kaulmann et al. (2014). Briefly, 0.1 g lettuce samples were extracted using 5% metaphosphoric acid. After centrifugation, the supernatant was mixed with 150 mmol/L sodium dihydrogen phosphate, 10% (w/v) trichloroacetic acid, 44% (w/v) orthophosphoric acid, 4% (w/v) dipyridine, and 3% (w/v) ferric chloride. The absorbance was measured at 525 nm for the calculation of AsA content. For measurement of reduced glutathione (GSH), the lettuce samples (0.1 g) were homogenized with 3% (v/v) trichloroacetic acid containing 5 mmol/L ethylenediaminetetraacetic acid. The GSH content was determined fluorometrically as reported by Owens and Belcher (1965).

2.6. Soluble sugar, free amino acids, and soluble protein analysis

Soluble sugar and free amino acids were measured according to the protocol of Wang et al. (2022b) with minor modifications. Lettuce samples (0.1 g) were homogenized in 1.5 mL 80% ethanol (v/v) and centrifuged at 10 000 g for 10 min at 4 °C. For soluble sugar determination, the extract was mixed with 5 mL 20 mg L^{-1} anthrone reagent prepared by sulfuric acid, and then incubated at 100 °C for 10 min. The absorbance of mixture was determined at 630 nm after cooling to room temperature. The free amino acids content was determined using ninhydrin reagent method (Moore & Stein, 1954). The soluble protein content was measured by Bradford's (1976) method. Briefly, the extract was mixed with coomassie brilliant blue G-250 and the absorbance was recorded at 595 nm.

2.7. Antioxidant capacity analysis

Ferric reducing antioxidant power (FRAP) assay was used to measure the total antioxidant activities of lettuce (Kaulmann et al., 2014). 0.1 mL of extracts were homogenized in 2.9 mL FRAP reagent and allowed to incubation for 10 min at room temperature. The absorbance was read at 593 nm by a spectrophotometer using $FeSO_4{\cdot}7H_2O$ solution as the calibration. The DPPH radical scavenging activity assay was determined by mixing the extracts with DPPH solution, and after incubation at room temperature for 30 min, the mixtures was measured at 517 nm (Zlotek et al., 2014). Regarding ABTS radical scavenging activity, the extracts were mixed with ABTS solution and followed by incubation for 12 h at the room temperature. The absorbance of the mixture was recorded at 734 nm (Souza et al., 2022).

2.8. Mineral analysis

The concentration of mineral elements, such as P, K, Ca, Mg, S, Fe, Cu, Zn, and B, was determined according to method of Almuhayawi et al. (2021). Briefly, the oven-dried lettuce leaves (0.3 g) were digested with the mixture of HNO_3 and $HClO_4$ (3:1, v/v). Then, the elements content was quantified by an inductively coupled plasma mass

Table 1
Effects of H_2O_2 on concentrations of individual phenolic compound in two lettuce genotypes.

Phenolic compounds (μg g^{-1} FW)	Ziluoma		Lvluo	
	CK	H10	CK	H10
Phenolic acids				
Caftaric acid	35.7 ± 2.0b	39.9 ± 7.4a	27.7 ± 4.5c	35.6 ± 6.3b
Chlorogenic acid	57.7 ± 8.7b	85.0 ± 12.6a	28.5 ± 3.6c	33.1 ± 5.8c
Coumaroylquinic acid	22.3 ± 1.2b	28.7 ± 0.6a	14.9 ± 1.8d	20.0 ± 2.2c
Chicoric acid 1	68.0 ± 13.8a	64.4 ± 5.6a	25.5 ± 1.7b	26.2 ± 3.8b
Chicoric acid 2	41.6 ± 7.9b	46.2 ± 6.1a	21.3 ± 4.5d	30.0 ± 6.8c
Di-*O*-caffeoylquinic acid	89.7 ± 18.5b	105.1 ± 12.3a	45.9 ± 9.1d	64.7 ± 13.8c
Flavonoids				
Quercetin-3-*O*-malonylglucoside	85.9 ± 14.5b	98.4 ± 13.4a	59.5 ± 12.9d	72.9 ± 7.9c
Quercetin derivative 1	48.1 ± 2.7b	68.9 ± 4.8a	30.0 ± 2.1c	43.1 ± 3.9b
Quercetin derivative 2	15.9 ± 2.5b	19.0 ± 3.9a	12.6 ± 1.8c	13.8 ± 0.7c
Apigenin conjugate	34.3 ± 1.1b	40.9 ± 2.0a	27.2 ± 1.8c	31.9 ± 2.4b
Anthocyanin				
Cyanin-3-(6″-*O*-acetyl)-glucoside	76.0 ± 12.9b	83.4 ± 11.3a	nd	nd

Different letters within each row indicate significant difference for different treatments at $P < 0.05$.

spectrometry (ICP-MS, NexION 300X, Perkin Elmer, USA) and calculated as mg or μg g^{-1} dry weight (DW).

2.9. Statistical analysis

The data is presented as mean ± standard deviation (SD) with five independent repetitions and all analyzed statistically using DPS software (Tang & Zhang, 2013). The one-way analysis of variance (ANOVA) was used to determine the significant differences within treatments at $P < 0.05$ or $P < 0.01$. Pearson correlation analysis was carried out to estimate the correlation among parameters at $P < 0.05$.

3. Results

3.1. Effect of H_2O_2 on growth and photosynthesis parameters

The 5 to 20 mmol/L H_2O_2 application increased the shoot and root fresh weight, of which 10 mmol/L H_2O_2 addition showing the maximum improvement by 12.3% and 13.6% for Ziluoma, and 22.1% and 18.1% for Lvluo, respectively (Fig. 1a). However, compared with the control treatment, the 40 mmol/L H_2O_2 treatment significantly decreased the shoot and root fresh weight. After H_2O_2 application, no obvious differences were found for plant height and leaf number of Ziluoma and Lvluo, compared with the control treatment (Fig. S1). These results indicated that proper concentration of H_2O_2 treatment is beneficial for lettuce growth; thus, 10 mmol/L H_2O_2 was used in the following study.

The significantly higher photosynthetic parameters including Pn, Gs, Tr, and WUE were found in H_2O_2-treated two lettuce genotypes, which increased by 14.2%, 8.9%, 31.9%, and 25.4% in Ziluoma, and by 22.4%, 18.6%, 15.1%, and 11.0% in Lvluo, respectively, compared with the control treatment (Fig. 1b). Similarly, the concentrations of Chl a, Chl b, and total Chl significantly increased by 14.0%, 27.0%, and 17.9%, respectively, in Ziluoma after treatment with 10 mmol/L H_2O_2, compared with the control treatment (Fig. S2).

3.2. Effect of H_2O_2 on phenolic profiles

Eleven phenolic compounds, including 6 phenolic acids, 4 flavonoids, and 1 anthocyanin, were identified by HPLC analysis (Table 1). Except for chicoric acid 1, most of the identified phenolic compounds were improved by H_2O_2 treatment in Ziluoma. Similarly, H_2O_2 treatments significantly enhanced the caftaric acid, coumaroylquinic acid, chicoric acid 2, di-*O*-caffeoylquinic acid, quercetin-3-*O*-malonylglucoside, quercetin derivatives 1, and apigenin conjugate contents by 28.5%, 34.35%, 40.8%, 40.8%, 22.9%, 43.9%, and 17.2% in Lvluo, respectively. Regardless of the treatments, the Ziluoma always showed the higher accumulation of the individual phenolic compounds than that in Lvluo.

3.3. Effect of H_2O_2 on phenolic content and phenolic biosynthesis enzymes

Compared with the control treatment, the total phenolic content significantly enhanced by 51.3% and 20.0%, respectively, in Ziluoma and Lvluo, after H_2O_2 treatment (Fig. 2a). Similar increases in total flavonoids (by 107.8% and 103.6%, respectively) and anthocyanin content (by 79.4% and 108.4%, respectively) were also observed in H_2O_2-treated Ziluoma and Lvluo, respectively. Regardless of the treatments, the higher contents of total phenolic, flavonoid, and anthocyanin were always shown in Ziluoma than that in Lvluo. PAL activity significantly increased by 11.0% and 13.3% in Ziluoma and Lvluo, respectively, after H_2O_2 treatment (Fig. 2b).

The transcript levels of genes encoded related enzymes (PAL, CHS, F3H, DFR, F35H, and UFGT) were determined in two lettuce genotypes (Fig. 2c and d). The significantly increases of most genes related to the phenolic biosynthetic pathway were observed after H_2O_2 treatment. The H_2O_2 treatment significantly up regulated the genes expression of PAL by 82.7% and 59.0%, CHS by 67.7% and 35.3%, F3H by 129.2% and 107.5%, DFR by 59.8% and 176.7%, and UFGT by 86.1% and 40.4% in Ziluoma and Lvluo, respectively, compared with the control treatment. Except for the F35H, the genes investigated in present study always showed the higher expression levels in Ziluoma than that in Lvluo, regardless of the treatments.

3.4. Effect of H_2O_2 on AsA, GSH, carotenoids, soluble sugar, soluble protein, and free amino acids

The higher concentrations of AsA, GSH, carotenoids, soluble sugar, soluble protein, and free amino acid were also observed in the H_2O_2-treated lettuce, which enhanced by 23.6%, 137.9%, 22.2%, 19.5%, 13.3%, and 23.2%, respectively, in Ziluoma (Fig. 3). In Lvluo, H_2O_2 application significantly increased the concentrations of GSH, carotenoids, soluble sugar, soluble protein, and free amino acid by 52.4%, 10.5%, 25.8%, 15.8%, and 15.2%, respectively, compared with those in control.

3.5. Effect of H_2O_2 on mineral contents

As shown in Table. 2, the elemental composition in the leaves of two lettuce genotypes was obviously affected by the H_2O_2 treatment. As compared with control treatment, the K, Ca, Mg, Fe, Cu, Zn, and B contents were significantly increased in both of two lettuce genotypes treated with H_2O_2. In addition, the H_2O_2 treatment also significantly improved the S content by 33.2% in Ziluoma and P content by 19.6% in Lvluo, respectively.

3.6. Effect of H_2O_2 on antioxidant capacities

Compared with the control treatment, the H_2O_2 treatment

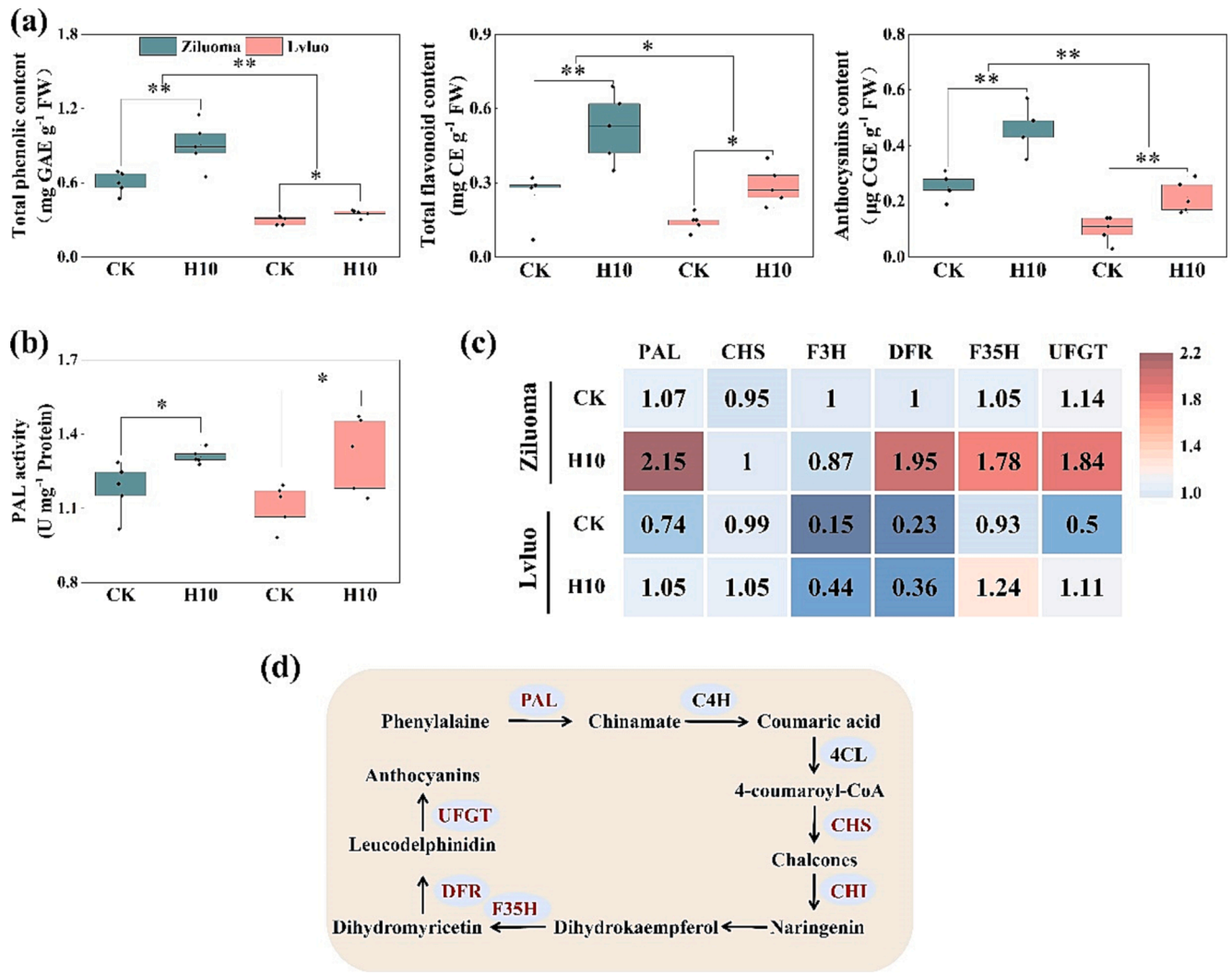

Fig. 2. Effect of H_2O_2 on the content of phenolic compounds and enzymes related to phenolic biosynthesis in lettuce plants. (a) Total phenolic, total flavonoid, and anthocyanin contents; (b) Phenylalanine ammonia-lyase (PAL) activity; (c) Heat map showing the relative gene expression levels of enzymes involved in phenolic biosynthesis, including PAL, chalcone synthase (CHS), flavanone 3-hydroxylase (F3H), dihydroflavonol-4-reductase (DFR), flavonoid 3′, 5′-hydroxylase (F35H), and UDP-glucose: flavonoid 3-O-glucosyltransferase (UFGT) in two lettuce genotypes; (d) Critical enzymes in the phenolic biosynthesis pathways. * and ** indicate significant differences between treatment with $P < 0.05$ and $P < 0.01$, respectively.

significantly improved the DPPH, FRAP, and ABTS by 15.1%, 36.5%, and 30.1%, respectively, in Ziluoma, and by 4.1%, 9.1%, and 21.3%, respectively, in Lvluo (Fig. 4a). The Ziluoma always had the higher values of DPPH, FRAP, and ABTS than that in Lvluo. The correlation matrix showed that the antioxidant substances, including phenolic compounds, AsA, GSH, and carotenoids generally showed a positive correlation with three antioxidant parameters (Fig. 4b and c). Among them, the total phenolic and flavonoid showed higher correlation coefficients with the antioxidant activity, which indicated that these compounds might have higher antioxidant activity than other antioxidants. In addition, correlation analysis for Ziluoma genotype represents that shoot fresh weight showed significantly positive correlation with chlorophyll content, most of bioactive constituents (total anthocyanin, GSH, soluble sugar, soluble protein, and free amino acid), and ABTS (Fig. 4b). Chlorophyll concentration and photosynthetic parameters were positively correlated with phenolic compounds, mineral nutrition, and antioxidant activity. In Lvluo genotype, the total chlorophyll content had negative correlation with bioactive constituents, mineral nutrition, and antioxidant activity (Fig. 4c).

4. Discussion

Elicitation with biotic or abiotic elicitors has been considered as a promising technique to improve accumulation of bioactive compounds and nutritional value of vegetables, although the yield usually reduced significantly (Liu et al., 2019; Zlotek et al., 2014). The H_2O_2 is now gaining more attentions as a potential signaling molecule in regulating plant physiological and biochemical reactions (Considine & Foyer, 2021). Therefore, it is interesting to determine the trade-off between the yield production and quality enhancement of vegetables by H_2O_2 elicitation. In present study, foliar spray of proper concentration of H_2O_2 obviously increased the content of bioactive compounds mainly including phenolic compounds, mineral elements, other antioxidants, and antioxidant capacity in two genotypes of lettuce without biomass loss. These results indicated that elicitation with H_2O_2 could be an effective method to produce vegetables with nutritional and healthy phytochemicals and high yield.

The roles of lower levels of H_2O_2 in regulating plant growth and development under desirable and stressful conditions have been shown. Studies have widely found the positive impacts of H_2O_2 in increasing the plant growth by alleviating the arsenic damage in rice (*Oryza sativa* L.),

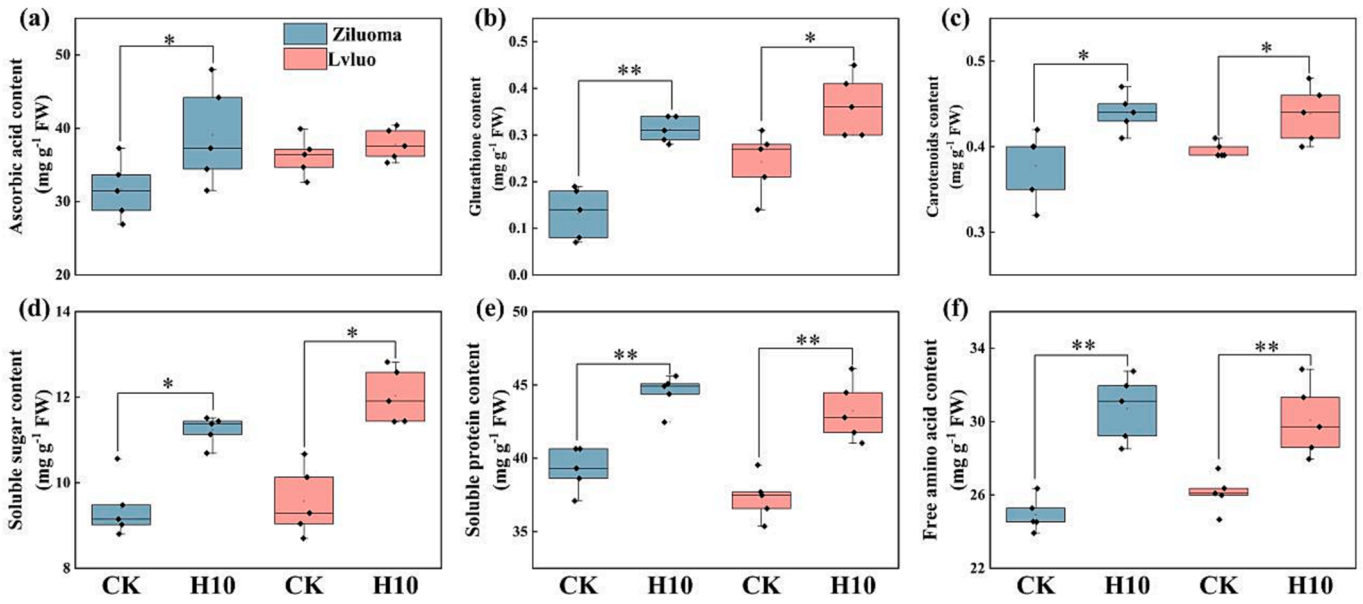

Fig. 3. Effect of H_2O_2 on the content of (a) ascorbic acid (AsA), (b) reduced glutathione (GSH), (c) carotenoids, (d) soluble sugar, (e) soluble protein, and (f) free amino acid in two lettuce genotypes. * and ** indicate significant differences between treatment with $P < 0.05$ and $P < 0.01$, respectively.

Table 2
Effects of H_2O_2 on mineral contents in two genotypes of lettuce leaves.

Mineral elements	Ziluoma		Lvluo	
	CK	H10	CK	H10
Macronutrient (mg g^{-1} DW)				
P	0.61 ± 0.11a	0.63 ± 0.19a	0.51 ± 0.09b	0.61 ± 0.13a
K	5.46 ± 0.52b	6.05 ± 0.74a	5.38 ± 0.93b	6.11 ± 0.61a
Ca	0.77 ± 0.11b	0.89 ± 0.16a	0.70 ± 0.11b	0.83 ± 0.13a
Mg	0.20 ± 0.05b	0.30 ± 0.04a	0.17 ± 0.06b	0.22 ± 0.04a
S	0.17 ± 0.04b	0.23 ± 0.04a	0.10 ± 0.01a	0.10 ± 0.02a
Micronutrient (μg g^{-1} DW)				
Fe	14.27 ± 2.71b	23.08 ± 5.41a	9.24 ± 1.30b	16.16 ± 2.29a
Cu	0.66 ± 0.05b	0.86 ± 0.07a	0.56 ± 0.03b	0.66 ± 0.06a
Zn	13.07 ± 1.50b	15.68 ± 1.08a	10.63 ± 1.27b	12.62 ± 1.17a
B	5.95 ± 0.82b	6.63 ± 0.44a	6.38 ± 0.24b	6.99 ± 0.19a

Different letters within each lettuce genotype indicate significant difference for different treatments at $P < 0.05$.

triclosan stress in wheat (*Triticum aestivum* L.), copper stress in tomato (*Solanum lycopersicum* L.), and nutrient deficiency in lettuce (*Lactuca sativa* L.), etc. (Asgher et al., 2021; Liu et al., 2020; Nazir et al., 2019). Moreover, in lentil sprouts, one step elicitation with 15 mmol/L H_2O_2 did not obviously reduce sprout yield but increase accumulation of antioxidants under normal conditions (Swieca, 2015). The current study showed that the 10 mmol/L H_2O_2 application significantly increased the shoot fresh weight of Ziluomo and Lvluo (Fig. 1a). This could be attributed to the well photosynthetic performance, which resulted in high supply of carbon resources for plant growth after H_2O_2 application. Here, the higher or consistent concentrations of chlorophyll *a*, chlorophyll *b*, and total chlorophyll were found in H_2O_2-treated Ziluoma and Lvluo (Fig. S2). Moreover, H_2O_2 addition significantly enhanced gas exchange attributes, including Pn, Gs, Tr, and WUE (Fig. 1b), suggesting that it induced improved CO_2 fixation and photosynthetic efficiency. It has also been found that H_2O_2 facilitates the photosynthetic efficiency by stimulating the Rubisco activity, Calvin cycle, and sugar metabolism, and thereby improving plant growth and yield (Asgher et al., 2021; Nazir et al., 2019).

Dietary phenolic compounds, including phenolic acids, flavonoids, and anthocyanins, have become a topic of increasing interest in recent years due to their various benefits on human health (Rashmi & Negi, 2020; Wang et al., 2020). The present study found that H_2O_2-cuased significantly increased accumulation of the total phenolic, flavonoids, anthocyanins, and most of the individual phenolic compounds (Fig. 2a and Table 1), and these enhancements resulting in a stronger antioxidant capacity reflected by DPPH, FRAP, and ABTS values (Fig. 4a). These results are consistent with the previous studies, which also found that H_2O_2 application could induce the biosynthesis of phenolic compounds with antioxidant function in vegetables (Khandaker et al. 2012; Liu et al., 2019; Swieca, 2015). Phenolic compounds have been demonstrated to exhibit greater antioxidant activity than vitamin C and E because of their chemical structure of numerous hydroxyl groups connected to the benzene ring (Daryanavard et al., 2023; Wang et al., 2020). Thus, we speculated that the phenolic compounds were the main contributors to the enhancement of antioxidant activity in H_2O_2-treated lettuce. The higher correlations between DPPH, ABTS and DPPH values and phenolic content also proved this viewpoint (Fig. 4b and c). The biosynthesis of phenolic compounds in lettuce is also influenced by the genotypic factors, and the higher accumulation of phenolic compounds generally presented in red leaf lettuces than in green lettuce types (Kim et al., 2016; Souza et al., 2022). Similarly, the content of phenolic compounds and antioxidant capacity in Ziluoma was obviously higher than that in Lvluo (Fig. 2a and Fig. 4a). Therefore, producing lettuce with higher content of phenolic compounds can be achieved by either manipulating cultivation conditions or choosing lettuce genotypes.

The H_2O_2 as a potential signaling molecule and plays a key role in regulating plant defense reactions (Considine & Foyer, 2021). Phenolic compounds, as an important group of antioxidants, are major components of plant antioxidant system (Brunetti, Di Ferdinando, Fini, Pollastri, & Tattini, 2013; Daryanavard et al., 2023). Previous studies have found that the improvement of phenolic accumulation is important in

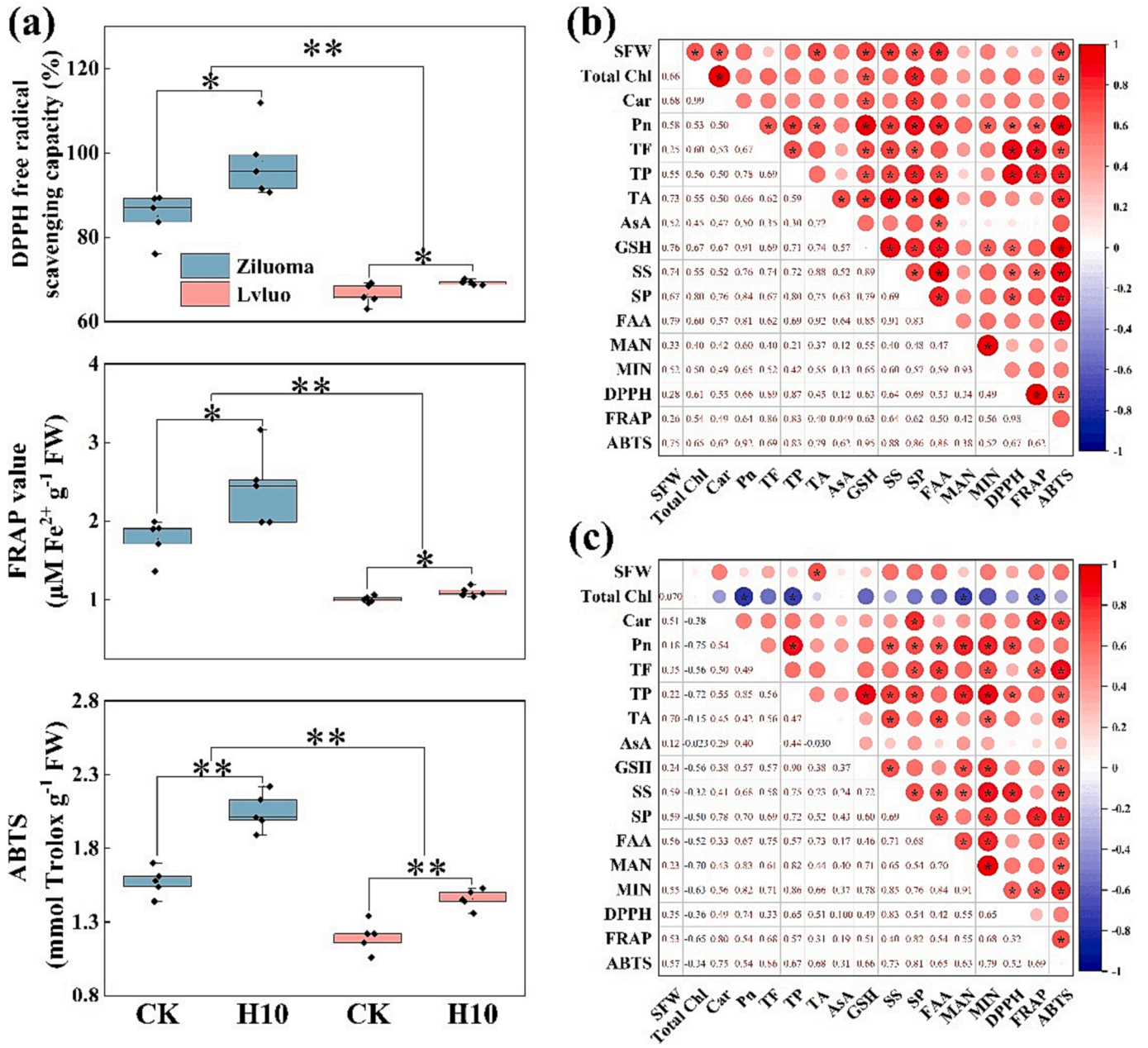

Fig. 4. Effect of H_2O_2 on antioxidant capacity (a) and the correlation of growth, photosynthetic parameters, quality parameters, and antioxidant activities in Ziluoma (b) and Lvluo (c). * and ** indicate significant differences between treatment with $P < 0.05$ and $P < 0.01$, respectively. Red and blue color represent positive and negative correlation, respectively. The large and small circular sizes correspond to strong and weak correlations, respectively. SFW, shoot fresh weight; Total Chl, total chlorophyll content; Car, carotenoids content; Pn, net photosynthetic; TF, total phenolic content; TF, total flavonoid content; TA, total anthocyanin content; AsA, ascorbic acid content; GSH, glutathione content; SS, soluble sugar content; SP, soluble protein content; FAA, free amino acid content; MAN, macronutrient (P, K, Ca, and S) contents; MIN, micronutrient (Fe, Cu, Zn, and B) contents; DPPH, DPPH scavenging capacity; FRAP, ferric reducing antioxidant power; ABTS, ABTS radical scavenging activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

protecting plants against oxidative damages (Daryanavard et al., 2023; Sun et al., 2021). In a feedback loop, H_2O_2 signaling can induce the biosynthesis of phenolic compounds by stimulating multiple genes involved in phenylpropanoid biosynthesis (Sun et al., 2021). Here, our results indicated that H_2O_2 elicitation increased the PAL activity and de novo biosynthesis of phenolic compounds by upregulating the transcriptional levels of main structural genes, including PAL, CHS, F3H, DFR, and UFGT involved in phenylpropanoid metabolism (Fig. 2b-d). These results imply that there may be cross-talk between H_2O_2 and phenolic accumulation in lettuce. In addition, the phenylpropanoid metabolism is closely related to the carbohydrate metabolism through the shikimate pathway, and thus the biosynthesis of phenolic compounds requires large number of carbon sources (Brunetti et al., 2013). It was reported that the changes in phenolic compounds induced by stress elicitors might be due to the redirection of the metabolic flux from nitrogen- to carbon-based metabolites (Caretto, Linsalata, Colella, Mita, & Lattanzio, 2015; Zhou et al., 2021). In our study, the H_2O_2 elicitation enhanced the chlorophyll content and photosynthetic efficiency (Fig. S2 and Fig. 1b), which potentially provide more carbon sources for the phenolic biosynthesis. Hence, it may be said that the H_2O_2 enhanced the supply of carbon resources by optimizing photosynthesis, subsequently increasing the phenolic biosynthesis (Asgher et al., 2021; Nazir et al., 2019).

The AsA, GSH, and carotenoids are small molecule antioxidants

widely exist in vegetables and play pivotal role in human health and normal life activities (Kim et al., 2016). Our results showed that H_2O_2 elicitation significantly increased the contents of AsA, GSH, and carotenoids in Ziluoma, and GSH and carotenoids content in Lvluo (Fig. 3). Correlation analysis also indicated the positive correlation between AsA, GSH, carotenoids contents and antioxidant activity (Fig. 4). These findings are in agreement with those results obtained in cauliflower, tomato, and mustard (Ellouzi, Oueslati, Hessini, Rabhi, & Abdelly, 2021; Khan, Khan, Masood, Per, & Asgher, 2016; Nazir et al., 2019). In addition, the higher levels of soluble sugar, soluble protein, and free amino acid were also observed in lettuce after H_2O_2 treatment under the present study (Fig. 3), which potentially providing more dietary carbohydrates and amino acid for human body. The higher photosynthetic efficiency in H_2O_2-treated lettuce may promote the accumulation of carbon and nitrogen metabolites. Dietary minerals, as an integral part of the human body, are also associated with the maintenance of health and metabolism, and thus increased intake is recommended. Lettuce is an important source of biologically effective minerals such as K, Ca, Mg, Zn, Cu, and Fe (Collado-Gonzalez et al., 2022; Kim et al., 2016). In this study, we observed that most of the mineral contents in H_2O_2 treatment was higher than these in CK treatment (Table 2). In accordance with our results, the significantly increased mineral content were also reported after application of H_2O_2 in several previous studies (Khandaker et al., 2012; Shin & Schachtman, 2004). This may be due to the enhanced root biomass in H_2O_2-treated lettuce (Fig. 1a), which increasing the mineral uptake by plants.

5. Conclusions

Elicitation with 10 mmol/L H_2O_2 showed the maximum impacts in maintaining biomass production, and improved enrichment of antioxidants mainly including phenolic compounds, mineral nutrients, and antioxidant capacity in lettuce. The H_2O_2 elicitation significantly induced accumulation of phenolic compounds by up-regulating enzymes activity and genes in the pathway of phenolic biosynthesis, while these impacts occurred in a genotype-dependent manner. This also may be due to the enhanced photosynthesis, which provides more carbon resources for the accumulation of phenolic compounds. Meanwhile, H_2O_2 also improved the nutritional value of lettuce by increasing the levels of AsA, GSH, carotenoids, soluble sugar, soluble protein, free amino acids, and minerals. Concomitantly, the antioxidant capacity has been improved as indicated by DPPH, ABTS, and DPPH values. Therefore, our study could help to apply H_2O_2 elicitation for the development of lettuce with health promoting components and high biomass production.

CRediT authorship contribution statement

Weixuan Wang: Methodology, Data curation, Writing – original draft, Funding acquisition. **Zikun Lin:** Methodology. **Weiran Wang:** Methodology. **Meixin Shang:** Methodology, Visualization. **Haofeng Lv:** Visualization, Writing – review & editing. **Quanli Zong:** Funding acquisition, Writing - review & editing. **Junliang Li:** Writing – review & editing. **Bin Liang:** Writing – review & editing. **Weiwei Zhou:** Conceptualization, Methodology, Data curation, Visualization, 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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Appendix A. Supplementary data

Supplementary data (Table S1. Sequences of primers used for the quantitative real-time PCR analysis. Fig. S1. Effect of H_2O_2 on plant height (a) and leaf number (b) in two lettuce genotypes. Different letters indicate significant difference at $P<0.05$. Fig. S2. Effect of H_2O_2 on chlorophyll a (a), chlorophyll b (b), and total chlorophyll (c) in two lettuce genotypes. * indicates significant differences between treatment with $P<0.05$.) to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100847.

References

Almuhayawi, M. S., Hassan, A. H. A., Al Jaouni, S. K., Alkhalifah, D. H. M., Hozzein, W. N., Selim, S., … Khamis, G. (2021). Influence of elevated CO2 on nutritive value and health-promoting prospective of three genotypes of Alfalfa sprouts (*Medicago Sativa*). *Food Chemistry, 340*, Article 128147.

Arnon, D. A. (1949). Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta Vulgaris*. *Plant Physiology, 24*, 1–15.

Asgher, M., Ahmed, S., Sehar, Z., Gautam, H., Gandhi, S. G., & Khan, N. A. (2021). Hydrogen peroxide modulates activity and expression of antioxidant enzymes and protects photosynthetic activity from arsenic damage in rice (*Oryza sativa* L.). *Journal of Hazardous Materials, 401*, Article 123365.

Bahcesular, B., Yildirim, E. D., Karaçocuk, M., Kulak, M., & Karaman, S. (2020). Seed priming with melatonin effects on growth, essential oil compounds and antioxidant activity of basil (*Ocimum basilicum* L.) under salinity stress. *Industrial Crops and Products, 146*, Article 112165.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Brunetti, C., Di Ferdinando, M., Fini, A., Pollastri, S., & Tattini, M. (2013). Flavonoids as antioxidants and developmental regulators: Relative significance in plants and humans. *International Journal of Molecular Sciences, 14*, 3540–3555.

Caretto, S., Linsalata, V., Colella, G., Mita, G., & Lattanzio, V. (2015). Carbon fluxes between primary metabolism and phenolic pathway in plant tissues under stress. *International Journal of Molecular Sciences, 16*, 26378–26394.

Collado-Gonzalez, J., Pinero, M. C., Otalora, G., Lopez-Marin, J., & Del Amor, F. M. (2022). Unraveling the nutritional and bioactive constituents in baby-leaf lettuce for challenging climate conditions. *Food Chemistry, 384*, Article 132506.

Considine, M. J., & Foyer, C. H. (2021). Oxygen and reactive oxygen species-dependent regulation of plant growth and development. *Plant Physiology, 186*, 79–92.

Daryanavard, H., Postiglione, A. E., Muhlemann, J. K., & Muday, G. K. (2023). Flavonols modulate plant development, signaling, and stress responses. *Current Opinion in Plant Biology, 72*, Article 102350.

Ellouzi, H., Oueslati, S., Hessini, K., Rabhi, M., & Abdelly, C. (2021). Seed-priming with H_2O_2 alleviates subsequent salt stress by preventing ROS production and amplifying antioxidant defense in cauliflower seeds and seedlings. *Scientia Horticulturae, 288*, Article 110360.

Jia, Z., Tang, M., & Wu, J. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry, 64*, 555–559.

Kaulmann, A., Jonville, M. C., Schneider, Y. J., Hoffmann, L., & Bohn, T. (2014). Carotenoids, polyphenols and micronutrient profiles of *Brassica oleraceae* and plum varieties and their contribution to measures of total antioxidant capacity. *Food Chemistry, 155*, 240–250.

Khan, M. I., Khan, N. A., Masood, A., Per, T. S., & Asgher, M. (2016). Hydrogen peroxide alleviates nickel-inhibited photosynthetic responses through increase in use-efficiency of nitrogen and sulfur, and glutathione production in mustard. *Frontiers on Plant Science, 7*, 44.

Khandaker, M. M., Boyce, A. N., & Osman, N. (2012). The influence of hydrogen peroxide on the growth, development and quality of wax apple (Syzygium samarangense, [Blume] Merrill & L.M. Perry var. *jambu madu*) fruits. *Plant Physiology and Biochemistry, 53*, 101–110.

Kim, M. J., Moon, Y., Tou, J. C., Mou, B., & Waterland, N. L. (2016). Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). *Journal of Food Composition and Analysis, 49*, 19–34.

Kitazaki, K., Fukushima, A., Nakabayashi, R., Okazaki, Y., Kobayashi, M., Mori, T., … Kusano, M. (2018). Metabolic reprogramming in leaf lettuce grown under different light quality and intensity conditions using narrow-band LEDs. *Scientific Reports, 8*, Article 7914.

Leon-Lopez, L., Escobar-Zuniga, Y., Salazar-Salas, N. Y., Mora Rochin, S., Cuevas-Rodriguez, E. O., Reyes-Moreno, C., & Milan-Carrillo, J. (2020). Improving polyphenolic compounds: Antioxidant activity in chickpea sprouts through elicitation with hydrogen peroxide. *Foods, 9*, Article 1791.

Lim, J. H., Park, K. J., Kim, B. K., Jeong, J. W., & Kim, H. J. (2012). Effect of salinity stress on phenolic compounds and carotenoids in buckwheat (*Fagopyrum esculentum* M.) sprout. *Food Chemistry, 135*, 1065–1070.

Lillo, C., Lea, U. S., & Ruoff, P. (2008). Nutrient depletion as a key factor for manipulating gene expression and product formation in different branches of the flavonoid pathway. *Plant Cell Environmental, 31*, 587–601.

Liu, H., Kang, Y., Zhao, X., Liu, Y., Zhang, X., & Zhang, S. (2019). Effects of elicitation on bioactive compounds and biological activities of sprouts. *Journal of Functional Foods, 53*, 136–145.

Liu, L., Huang, L., Lin, X., & Sun, C. (2020). Hydrogen peroxide alleviates salinity-induced damage through enhancing proline accumulation in wheat seedlings. *Plant Cell Reports, 39*, 567–575.

Moore, S., & Stein, W. H. (1954). A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. *Journal of Biological Chemistry, 211*, 907–913.

Nazir, F., Hussain, A., & Fariduddin, Q. (2019). Hydrogen peroxide modulate photosynthesis and antioxidant systems in tomato (*Solanum lycopersicum* L.) plants under copper stress. *Chemosphere, 230*, 544–558.

Owens, C. W. I., & Belcher, R. V. (1965). A colorimetric micro-method for the determination of glutathione. *Biochemistry Journal, 94*, 705–711.

Rashmi, H. B., & Negi, P. S. (2020). Phenolic acids from vegetables: A review on processing stability and health benefits. *Food Research International, 136*, Article 109298.

Shin, R., & Schachtman, D. P. (2004). Hydrogen peroxide mediates plant root cell response to nutrient deprivation. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 8827-8832.

Souza, A. S. N., Schmidt, H. O., Pagno, C., Rodrigues, E., Silva, M., Flores, S. H., & Rios, A. O. (2022). Influence of cultivar and season on carotenoids and phenolic compounds from red lettuce influence of cultivar and season on lettuce. *Food Research International, 155*, Article 111110.

Sun, H., Cao, X., Wang, X., Zhang, W., Li, W., Wang, X., … Lyu, D. (2021). RBOH-dependent hydrogen peroxide signaling mediates melatonin-induced anthocyanin biosynthesis in red pear fruit. *Plant Science, 313*, Article 111093.

Swieca, M. (2015). Production of ready-to-eat lentil sprouts with improved antioxidant capacity: Optimization of elicitation conditions with hydrogen peroxide. *Food Chemistry, 180*, 219–226.

Tang, Q.-Y., & Zhang, C.-X. (2013). Data Processing System (DPS) software with experimental design, statistical analysis and data mining developed for use in entomological research. *Insect Science, 20*, 254–260.

Wang, M., Leng, C., Zhu, Y., Wang, P., Gu, Z., & Yang, R. (2022). UV-B treatment enhances phenolic acids accumulation and antioxidant capacity of barley seedlings. *LWT-Food Science and Technology, 153*, Article 112445.

Wang, W., Zhang, C., Shang, M., Lv, H., Liang, B., Li, J., & Zhou, W. (2022a). Hydrogen peroxide regulates the biosynthesis of phenolic compounds and antioxidant quality enhancement in lettuce under low nitrogen condition. *Food Chemistry-X, 16*, Article 100481.

Wang, W., Zhang, C., Zheng, W., Lv, H., Li, J., Liang, B., & Zhou, W. (2022b). Seed priming with protein hydrolysate promotes seed germination via reserve mobilization, osmolyte accumulation and antioxidant systems under PEG-induced drought stress. *Plant Cell Reports, 41*, 2173–2186.

Wang, Z., Li, S., Ge, S., & Lin, S. (2020). Review of distribution, extraction methods, and health benefits of bound phenolics in food plants. *Journal of Agricultural and Food Chemistry, 68*, 3330–3343.

Yang, X., Hu, J., Wang, Z., Huang, T., Xiang, Y., Zhang, L., … Yang, Q. (2023). Pre-harvest nitrogen limitation and continuous lighting improve the quality and flavor of lettuce (*Lactuca sativa* L.) under hydroponic conditions in greenhouse. *Journal of Agricultural and Food Chemistry, 71*, 710–720.

Zhou, W., Chen, Y., Xu, H., Liang, X., Hu, Y., Jin, C., … Lin, X. (2018). Short-term nitrate limitation prior to harvest improves phenolic compound accumulation in hydroponic-cultivated lettuce (*Lactuca sativa* L.) without reducing shoot fresh weight. *Journal of Agricultural and Food Chemistry, 66*, 10353–10361.

Zhou, W., Liang, X., Li, K., Dai, P., Li, J., Liang, B., … Lin, X. (2021). Metabolomics analysis reveals potential mechanisms of phenolic accumulation in lettuce (*Lactuca sativa* L.) induced by low nitrogen supply. *Plant Physiology and Biochemistry, 158*, 446–453.

Zlotek, U., Swieca, M., & Jakubczyk, A. (2014). Effect of abiotic elicitation on main health-promoting compounds, antioxidant activity and commercial quality of butter lettuce (*Lactuca sativa* L.). *Food Chemistry, 148*, 253–260.