Low temperature and high humidity affect dynamics of chlorophyll biosynthesis and secondary metabolites in Cucumber

Bakht Amin[1,2,3], Muhammad Jawaad Atif[4,5], WMWW Kandegama[6,7], Jamal Nasar[1], Pravej Alam[8], Zhongming Fang[1,2]* and Zhihui Cheng[3]*

Abstract

Background During the cold season, low temperature (LT) and high relative humidity (HRH) are significant environmental factors in greenhouses and plastic tunnels, often hindering plant growth and development. The chlorophyll (Chl) biosynthesis inhibitory mechanisms under LT and HRH stress are still widely unclear. To understand how cucumbers seedlings respond to LT and HRH stress, we investigated the impact of these stressors on Chl biosynthesis.

Results Our results revealed that individual LT, HRH and combined LT + HRH stress conditions affected chlorophyll *a*, *b*, total chlorophyll and carotenoid content, reducing the levels of these pigments. The levels of Chlorophyll precursors were also markedly reduced under LT and HRH stresses, with the greatest reduction observed in cucumber seedlings exposed to LT + HRH conditions (9/5 ℃, 95%HRH). The activities of glutamate-1-semialdehyde transaminase (GSA-AT), ALA dehydratase (ALAD), Mg-chelatase, and protochlorophyllide oxidoreductase (POR) were increased under individual LT, HRH, conditions but decreased by combination of LT + HRH stress condition. In addition, Chl biosynthesis related genes (except PBG) were upregulated by the HRH stress but were significantly downregulated under the LT + HRH stress condition in cucumber seedlings. Furthermore, the content of phenols, flavonoids and phenolic acids (cinnamic acid and caffeic acid) were significantly surged under LT + HRH treatment over the control. Histochemical observation showed higher O_2^- and H_2O_2 content in cucumber leaves during the LT and HRH stress.

Conclusion The results indicate that LT + HRH stress significantly impairs chlorophyll biosynthesis in cucumber seedlings by drastically reducing pigment accumulation, altering enzyme activity and gene expression. Additionally, LT + HRH stress induces oxidative damage, which further exacerbates the decline in chlorophyll content and affects overall cucumber metabolism.

Keywords *Cucumis sativus*, Low temperature, High humidity, Chlorophyll biosynthesis, Gene expression, Secondary metabolites

*Correspondence:
Zhongming Fang
zmfang@gzu.edu.cn
Zhihui Cheng
changzh@nwafu.edu.cn
Full list of author information is available at the end of the article

Introduction

Plants are exposed to several types of abiotic stresses, which effect their growth, development and productivity [1]. Cold stress/Low temperature (LT) hinders the growth and development of plants, leading to reduced yield, especially in vegetable crops like cucumbers [2]. Greenhouse vegetable production is common globally, however, fluctuation in temperature and humidity impede plant growth and development [3, 4].

Cold stress triggers an array of physiological disruptions in plants [4]. Physiologically, the primary biochemical process affected by LT stress is photosynthesis, which greatly influenced plant growth and leads to the decline of chlorophyll (Chl) biosynthesis [7]. Photosynthesis and chlorophyll pigments are linked with each other and highly sensitive to changes in temperature and humidity, which can significantly damage the photosynthetic processes in plant [2,8]. Plant photosynthetic performance is often measured using PSII's maximum photochemical efficiency (F_v/F_m) [9]. For measuring plant stress responses, it is claimed that the F_v/F_m ratio is a more reliable parameter than the F_v/F_0 [8]. Its optimal value is approximately 0.830, which is observed in most of the healthy plants [7], however in stressed plants, F_v/F_m values decrease, indicating possible damage to the PSII reaction Centre [7]. Nonphotochemical quenching (NPQ) plays a crucial role in protecting plants from oxidative damage in response to cold stress [9]. Notably, long exposure of cucumber leaves to low temperatures (4 ℃), coupled with photon flux densities approaching 200 mol m⁻²s⁻¹, lead to 50% reduction in photooxidisable P700 content [10]. Interestingly, this extended cold exposure exhibited only a marginal impact on PSII, as reported previously [11].

Plant activates their non-enzymatic and enzymatic antioxidative defense system when exposed to cold environment [46]. A wide range of secondary metabolites like phenylpropanoids and their derivatives are generated by plants in response to environmental cues [12, 13]]. These phenolic compounds are particularly notable for their strong ability to neutralize reactive oxygen species (ROS) [54]. The metabolites like coumarins, lignin building blocks, and flavonoids display semi-polar characteristics and play various physiological roles, including scavenging ROS and activating enzymes [16]. Phenolic acids such as caffeic acid, ferulic acid, cinnamic acid, and *p*-coumaric acid are known to contribute significantly to plant defense against various environmental stresses [17, 18].

Chl are crucial for collection and transmission of light energy in antenna systems and separation of charges and transport of electrons in reaction centers [16], and share a metabolic pathway with various tetrapyrroles (e.g., siroheme, heme, phytochromobilin) present in plant, algae, and bacteria [17]. Chl biosynthesis is an intricate process, characterized by a series of enzyme-catalyzed reactions, divided into distinct stages [8]. During the initial phase, Protoporphyrin IX (Proto IX) is synthesized from glutamate [18]. The initial and critical step in chlorophyll biosynthesis involves the generation of δ-aminolevulinic acid (ALA, the primary precursor), which is encoded by *HEMA* gene [16]. Porphobilinogen (PBG) gene results from the condensation of two molecules of ALA under the catalysis of ALA dehydratase (ALAD). Subsequently, Uroporphyrinogen III (urogen III) is produced by the polymerization of four PBGs, followed by cyclization, leading to the formation of coproporphyrinogen III (coprogen III) [19]. Protoporphyrin IX (Proto IX) is finally generated through the oxidative decarboxylation and oxygen-dependent aromatization of coprogen III [20]. The formation of chlorophyll *a* (Chl *a*) from Proto IX is an integral process in the second phase [21]. The *CAB* gene encodes a protein that is essential for the stability and function of light-harvesting complexes, which are crucial for photosynthesis [17]. LT cause Chl degradation, the leaves progressively turn yellow which represent carotenoid content [22]. Chl biosynthesis is a complex process mediated by various enzymes involved in chlorophyl formation [17]. One of the key enzymes involved is Mg-chelatase, which facilitates the conversion of Mg^{2+} and protoporphyrin IX into Mg porphyrin IX in plants [17]. Mg-chelatase comprises four essential components (H subunit, CHLH/ABAR; I subunit, CHLI; D subunit, CHLD; and GENOMES UNCOUPLED 4, GUN4 protein), which work together to control its activity during chlorophyll biosynthesis [22]. Abiotic stress such as LT alter *ChlH* expression, affecting the overall rate of chlorophyll biosynthesis, which is crucial for maintaining chlorophyll levels and photosynthetic capacity [23].

Cucumber (*Cucumis sativus*, Cucurbitaceae family) is a commonly grown creeping vine plant that produces typically cylindrical fruits used as vegetables [2]. Cucumber growth and development is affected by fluctuating environmental factors like temperatures and humidity [2, 6]. Although it has been demonstrated that LT severely inhibits formation of chlorophyll biosynthesis [17]. However, the inhibitory mechanism of chlorophyll biosynthesis in cucumber transplant under combination of LT and HRH stress has not been reported earlier. Understanding how LT and HRH stresses and inhibit chlorophyll formation in cucumber can offer valuable insights and genetic resources for the development of tolerance to both the stress conditions. Consequently, the present experiment was designed to examine the fundamental mechanisms governing chlorophyll biosynthesis in the presence of LT and HRH stress, providing valuable insights to improve cucumber production.

Methods

Plant material, growth conditions and stress treatments

In this study, the cucumber cultivar "shuyanbailv" was chosen as the experimental material, and the research was carried out in growth chamber (RGL-P800, Ningbo Jiangnan Instrument Factory, Zhejiang Province, China) at the College of Horticulture, Northwest A&F University, China as per protocols reported previously [2]. Once the cotyledons had completely open, uniform-sized seedlings were shifted into plastic pots filled with nutrient medium containing "Jiahui" (Liaocheng, Shandong Province, China), which is composed of 20–25% organic matter and 8–10% humic acid. Subsequently, the seedlings were subjected to different combinations of low-temperature (LT) and high relative humidity (HRH) treatment for 6 days. The four treatment conditions were as follows: control (CK: 25/18℃, 80% RH: relative humidity), control with high relative humidity (CK+HRH: 25/18℃, 95% HRH), low-temperature (LT: 9/5℃, 80% RH), and combination of low-temperature and high relative humidity (LT+HRH: 9/5℃, 95% HRH). Fresh leaf samples from each treatment within each replication were collected after the treatment, snap-frozen, and stored for subsequent analysis. The experiment was conducted in triplicate, each replication consisting of nine plants.

Measurement of morphological parameters

To determine shoot fresh weight, shoot dry weight, and total dry weight, electronic balance was employed. After these measurements, the samples were stored subsequently used for further analyses, including the determination of chlorophyll content and other metabolites. The strong seedling index (SSI) was calculated following the method previously described [25].

$$Strong\ Seedling\ Index\ (SSI) = \left(\frac{Stem\ diameter}{Plant\ height} + \frac{Root\ dry\ weight}{Shoot\ dry\ weight} \right) \times Total\ dry\ weight$$

Measurement of relative water content (RWC)

To assess RWC, samples were initially cut and their weight was promptly recorded. Subsequently, these leaf samples were rehydrated by immersing them hole night in water. The following day, the fresh weight was assessed before they were dried and RWC was calculated per the previous report [26].

$$RWC = \frac{(fresh\ weight - dry\ weight)}{(turgid\ weight - dry\ weight)} \times 100$$

Determination of chlorophyll pigments and carotenoids

The extraction of chlorophyll pigments and carotenoid content was carried out in acetone under dim light conditions, following the detailed protocol stated [2].

Determination of soluble proteins and δ-Aminolaevulinic acid

The protein concentration was assessed through a colorimetric approach, as described in detailed [15]. δ-Aminolevulinic acid (ALA) content was detected in accordance with the method described earlier [22].

Detection of chlorophyll precursors

Porphobilinogen (PBG) was extracted as previously reported [27]. Fresh leaf tissue (0.5 g) was pulverized in 5 mL of extraction solution containing (0.6 mol/L Tris, 0.1 mol/L EDTA, pH 8.2) on ice bath. The absorbance of the resulting solution was measured at 553 nm to determine PBG concentration [27].

The urogen III and coprogen III contents were measured by following the previously reported protocol [27]. A leaf sample (0.5 g) was homogenized in 10 mL of 0.067 mol/L PBS (pH 6.8) on ice. The homogenate was then centrifuged at $18,000 \times g$ for 10 min at 4 °C and subsequently the supernatant was recorded at 405.5 nm wavelength spectrophotometer. The Proto IX, Mg-proto IX, protochlorophyllide (Pchlide), and Mg-protoporphyrin monomethyl ester (Mpe) content was estimated as per the reported procedure [7, 27].

Analysis of Chlorophyll Biosynthesis enzymes

Cucumber leaf samples (0.5 g) were homogenized in phosphate buffer at 4 ℃. The activity of Glutamate 1-semialdehyde aminotransferase (GSA-AT) was measured according to previously reported detailed protocol [33].

The activity of ALA dehydratase (ALAD) was assessed following the protocol described by Tewari et al. [29].

Mg-chelatase activity was determined as described by Yaronskaya et al. [30]. The activity of POR was detected using the procedures of [28].

Measurement of secondary metabolites

Phenolic acids were measured using the method of Chen et al. [31]. Briefly, 1 g of leaf sample was ground in liquid nitrogen, mixed with 3 mL of methanol, and centrifuged for 15 min. The supernatants from two consecutive extractions were combined, dried under N2, and re-dissolved in 200 μL methanol. The free phenolic acids were extracted using cyclohexane/ethyl acetate and HCl, while the glycosidic-bound phenolic acids were released by incubation with β-glucosidase. The extracts were then filtered (0.45 μm) and analyzed by HPLC-MS.

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Flavonoids were extracted from 0.5 g of fresh cucumber leaves with 2 mL of methanol/HCl (99:1, v/v) and left to stand at room temperature for 12 h. For analysis, 300 µL of the supernatant was mixed with 5% NaNO2 and 10% AlCl3, and 2 mL of 1 N NaOH after centrifugation.

Lignin content was determined according to the method of Ali et al. [16]. Fresh leaf samples (0.3 g) were ground, mixed with 10 mL of 99.5% ethanol, and centrifuged for 15 min. The dried pellet was treated with 2 N HCl and 0.5 mL thioglycolic acid, followed by an 8-hour water bath. After cooling, the samples were centrifuged, and the supernatant was treated with 1 mL of HCl to precipitate lignin. The precipitate was dissolved in 1 N NaOH, and lignin content was measured electrometrically at 280 nm.

Measurement of O2– and H2O2 level
The levels of O_2^- and H_2O_2 were measured using the protocols described in previous studies [31, 33].

3,3′-Diaminobenzidine (DAB) and nitrotetrazolium blue chloride (NBT) staining
DAB and NBT staining were performed as described previously [33]. Leaf sample was immersed in DAB solution to detect H_2O_2 and in NBT solution to detect O_2^-, then incubated in the dark overnight. The samples were then allowed to cool at room temperature. H_2O_2 appeared as a brown stain, while O_2^- appeared as a dark blue stain [33].

Chlorophyll fluorescence parameters
The chlorophyll fluorescence parameters were detected utilizing multispectral fluorescence imaging technology (FC800 FluorCam, PSI Czech). To determine F_v/F_m, NPQ, qP, and ETR, the plants were placed in room with no light for 30 min. The fluorescence was observed by employing a pulsed light 0.8 s with an intensity of 4,000 µmol s^{-1} m^{-2}. The F_v/F_m ratio was calculated for each plant, with the entire leaf considered as the area of interest [34].

Extraction of total RNA and real-time quantitative PCR
RNA was isolated from leaves using the Total RNA Isolation Kit (Omega Bio-Tek). First-strand cDNA was synthesized from 1 µg of RNA with the PrimeScript RT Reagent Kit (Takara Bio). The resulting cDNA was then diluted to 200 µg/mL. Quantitative PCR was performed using the Bio-Rad CFX 134 Connect Real-Time PCR Detection System and a SYBR Green qPCR Kit. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method [35]. The primers Primer3web (version 4.1) was used to design the primers (version 4.1) (Supplementary Table 1).

Statistical analysis
Bartlett's test and Shapiro–Wilk test were used to assess the homogeneity of variance, independence of errors, and normality of distribution, ensuring that the data met the assumptions required for ANOVA (analysis of variance). The variables measured for different treatments were subjected to ANOVA with treatment as the independent variable. The triplicate data among treatments were analyzed using Statistic (version 8.1, Chicago, IL) and Tukey's HSD test were performed for significance differences. Differences were considered statistically significant when $P < 0.05$.

Results
Effect of LT and HRH on plant growth
Results of individual and combined low-temperature and high relative humidity stress on the growth of seedlings under CK (25/18℃, 80%), CK+HRH (25/18℃, 95%), low-temperature (LT: 9/5℃, 80%), and LT+HRH (9/5℃, 95%), conditions showed that leaves were green and fully expanded in the control condition (CK: 25/18℃, 80%) (Fig. 1). In contrast, slowed growth were observed in cucumber seedling under all other treatments compared to the control, and post-treatment the leaf margin slightly turns yellow. The whitish color significantly appeared in leaf blade of cucumber plant treated with LT (9/5℃, 80%), and LT+HRH (9/5℃, 95%), indicating that these
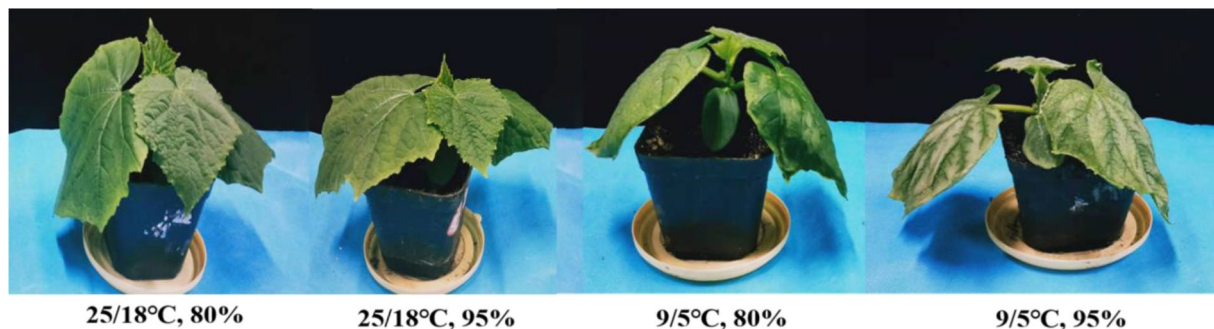
Fig. 1 Effect of low-temperature and high relative humidity stress on cucumber. CK: 25/18℃, 80% represent control condition, CK+HRH: 25/18℃, 95% represent control temperature and high relative humidity, LT: 9/5℃, 80% represent low-temperature and control humidity, LT+HRH: 9/5℃, 95% represent low-temperature and high relative humidity

two stress conditions inhibit chlorophyll biosynthesis (Fig. 1).

At 6-day post-treatment we measured the shoot fresh weight, shoot dry weight, SSI, total dry weight and RWC. These parameters were significantly reduced under high relative humidity (CK+HRH), low-temperature (LT) and low-temperature and high relative humidity LT+HRH stress, when compared to the plant grown under control (CK) environment (Table 1). No significant differences were observed under HRH alone, however LT and LT+HRH stress significantly reduced RWC. These results showed that LT and LT+HRH stress hindered the growth of cucumber.

Effect of LT and HRH on leaf pigments and carotenoid content

Data obtained at the 6th day post-treatment indicate that high relative humidity (CK+HRH) low-temperature (LT) and LT+HRH treatments significantly affected the chlorophyll pigments and corotenoid content of cucumber seedlings compared control plants (Fig. 2). The treated seedlings showed reduced levels of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid content, with the most pronounced reduction observed under the LT+HRH treatment.

Effect of LT and HRH on chlorophyll intermediates and protein in cucumber

Further analysis revealed that soluble protein content dramatically increased significantly under CK+HRH and LT treatment than conrol (CK). However, the LT+HRH treatment did not differ significantly from the control (Fig. 3A) (Fig. 3A). ALA, the first chlorophyll biosynthesis intermediate, showed reduced levels in the CK+HRH, LT and LT+HRH treatments, compared to the control, CK (Fig. 3B), which suggest that ALA biosynthesis was inhibited in cucumber seedlings under LT+HRH stress. PBG content also showed downward trend in response to CK+HRH, LT, while the LT+HRH showed levels similar to LT (Fig. 3C).

The Urogen III and Coprogen III content was compared in different conditions, and results revealed that their levels were reduced under LT and LT+HRH stresses, and no impact was observed in the CK+HRH condition, compared to the control plants (Fig. 3D and E).

The level of Proto IX decreased under CK+HRH, LT and LT+HRH treated plants than control plants (CK) (Fig. 3F). Mg-proto IX levels were declined in each treatment, particularly sharp decline was found in LT+HRH treated plants, compared to control (Fig. 3G). Mpe content was slightly decreased under CK+HRH treatment, while a drastic reduction was observed under LT and LT+HRH stress conditions (Fig. 3H). Analysis of Pchlide content in the leaves of cucumber plants showed lower levels, under CK+HRH and LT conditions. A more drastic reduction was observed under LT+HRH conditions compared to the control (Fig. 3I). In summary, LT and HRH stress hinders chlorophyll biosynthesis by impeding ALA synthesis and obstructing the conversion of Pchlide into Chls.

Effect of LT and HRH on chlorophyll biosynthesis enzyme activities

Next, we measured the activities of key enzymes involved in chlorophyll biosynthesis under low temperature and high relative humidity conditions (Fig. 4). Activity of glutamate-1-semialdehyde transaminase (GSA-AT, responsible for catalyzing the conversion of glutamate-1-semialdehyde into ALA), was elevated in response to CK+HRH, LT, and LT+HRH conditions compared to control (Fig. 4A). ALA dehydratase (ALAD) activity also upsurge under all the three condition with maximum levels in CK+HRH (Fig. 4B). Mg-chelatase (crucial enzyme that initiates the Mg-dependent segment of the chlorophyll biosynthetic pathway) activity also enhanced substantially in all the three stresses (maximum in LT) compared to control plants (Fig. 4C). The activity of POR enzyme that catalyzes the conversion of Pchlide to Chlide during chlorophyll biosynthesis, increased sharply under CK+HRH and LT, whereas LT+HH treatment showed levels similar to control (Fig. 4D). In short, the reduced levels of ALA may be ascribed to the combination of low-temperature and high relative humidity stress-induced inhibition of GSA-AT activity, and the impediment of the Pchlide to Chlide conversion is likely associated with diminished POR activity against combined low-temperature and high-humidity stress.

Table 1 Effect of low-temperature and high relative humidity stress on cucumber plant morphology

Treatment	Shoot fresh weight (g plant^{-1})	Shoot dry weight (g plant^{-1})	SSI	Total dry weight (g plant^{-1})	RWC (%)
25/18℃, 80%	10.11 ± 0.45[a]	0.83 ± 0.04[a]	0.83 ± 0.02[a]	1.03 ± 0.01[a]	85.93 ± 1.17[a]
25/18℃, 95%	9.10 ± 0.01[b]	0.70 ± 0.01[b]	0.82 ± 0.03[a]	1.02 ± 0.01[a]	84.49 ± 1.28[a]
9/5℃, 80%	8.79 ± 0.01[b]	0.47 ± 0.05[c]	0.64 ± 0.02[b]	0.83 ± 0.02[b]	41.60 ± 1.35[b]
9/5℃, 95%	8.13 ± 0.12[c]	0.37 ± 0.01[d]	0.52 ± 0.01[c]	0.67 ± 0.03[c]	36.76 ± 0.71[c]

Different letters represent significant differences while same letter represent non–significant differences. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, P<0.05
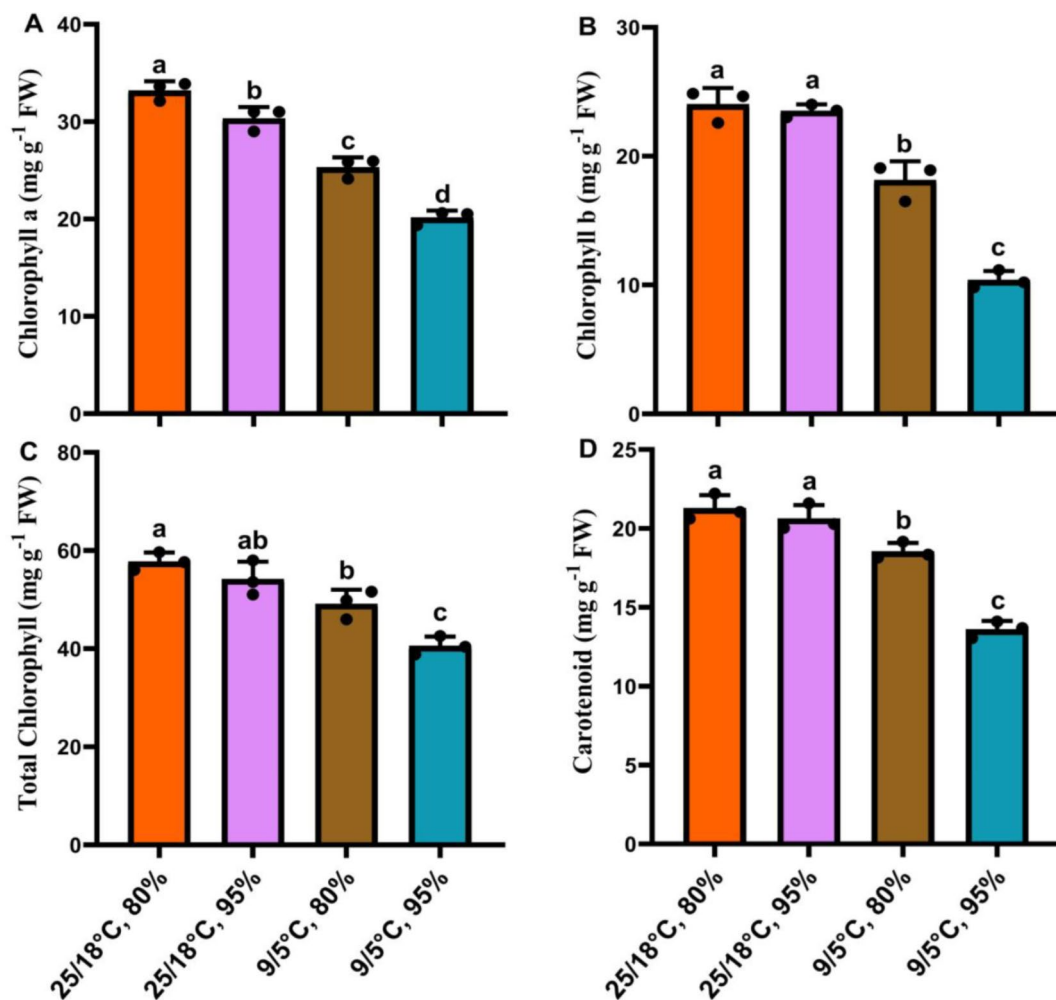
Fig. 2 Effect of low-temperature and high relative humidity on chlorophyll a (**A**), chlorophyll b (**B**), total chlorophyll (**C**), and carotenoid content (**D**) of cucumber seedlings. CK: 25/18℃, 80% represent control condition; CK+HRH: 25/18℃, 95% represent control temperature and high relative humidity; LT: 9/5℃, 80% represent low-temperature and control humidity; LT+HRH: 9/5℃, 95% represent low-temperature and high relative humidity combination. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $P < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment

Effects of LT and HRH on expression of chlorophyll biosynthesis associated genes

RT-qPCR analysis was done to assess elucidate the impact of low-temperature and high relative humidity on the expression levels of 4 pivotal chlorophyll biosynthesis genes. Results revealed that *CAB* and *ChlH* gene under CK+HRH treatment was significantly upregulated compared to control, while in LT and LT+HRH treatments the genes were downregulated (Fig. 5A, B) There was no significant difference in the expression of *PBG* gene between control and CK+HRH, whereas under LT and LT+HRH the gene was downregulated (Fig. 5C). In case of *POR* gene, the CK+HRH showed upregulation, while LT and LT+HRH showed downregulation compared to the control plants (Fig. 5D). The expression of *HEMA* increased under CK+HRH and LT treatments, while in

LT+HRH condition, the expression was down regulated (Fig. 5E).

Effect of LT and HRH on chlorophyll fluorescence parameters

Chlorophyll fluorescence images analysis revealed that CK+HRH, LT and LT+HRH conditions adversely affected on PSII system (Fig. 6A) and reduced F_v/F_m in cucumber compared to the control conditions (CK) (Fig. 6B). The non-photochemical quenching (NPQ) content exhibited a significant increase in response to both individual and combined treatments (Fig. 6C), whereas the photochemical quenching (qP) and electron transport rate (ETR) displayed an inverse trend (Fig. 6D, E), with lowest values in plants subjected LT+HRH treatments.
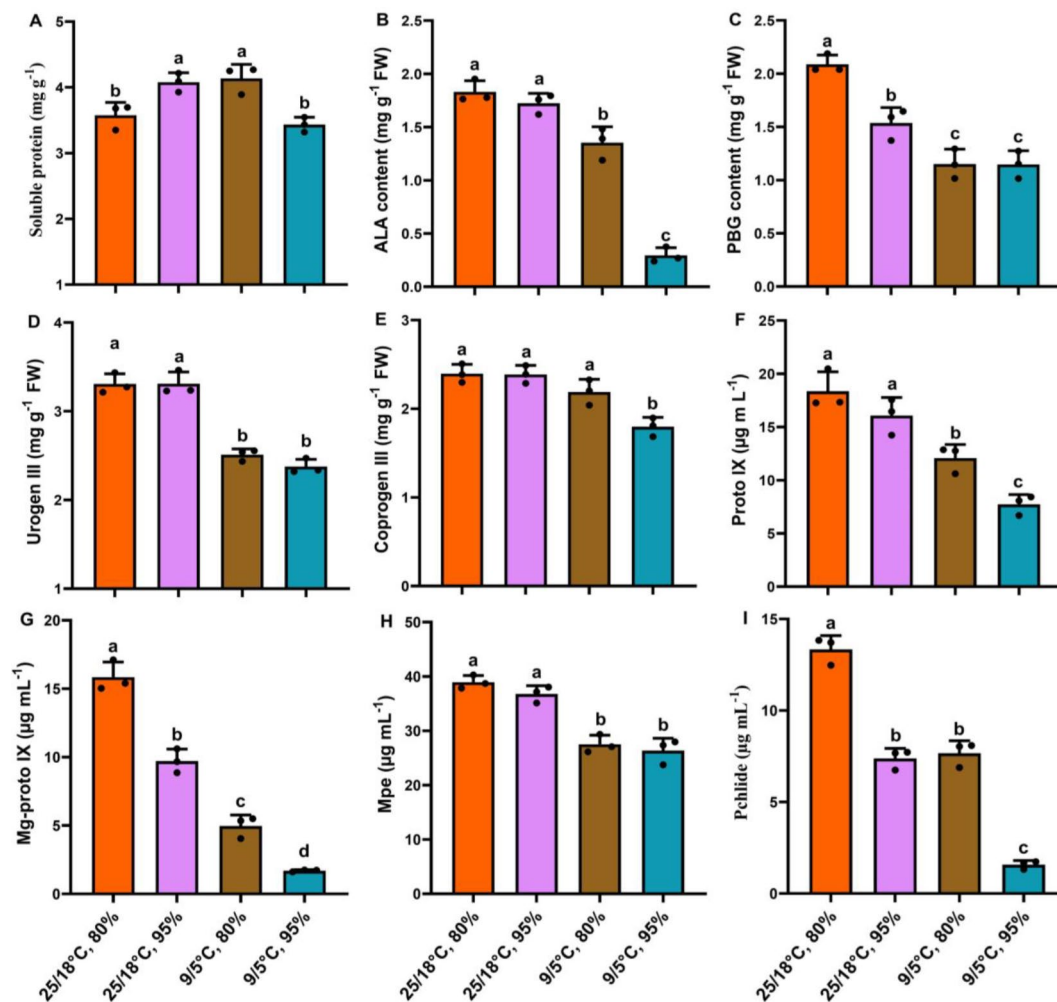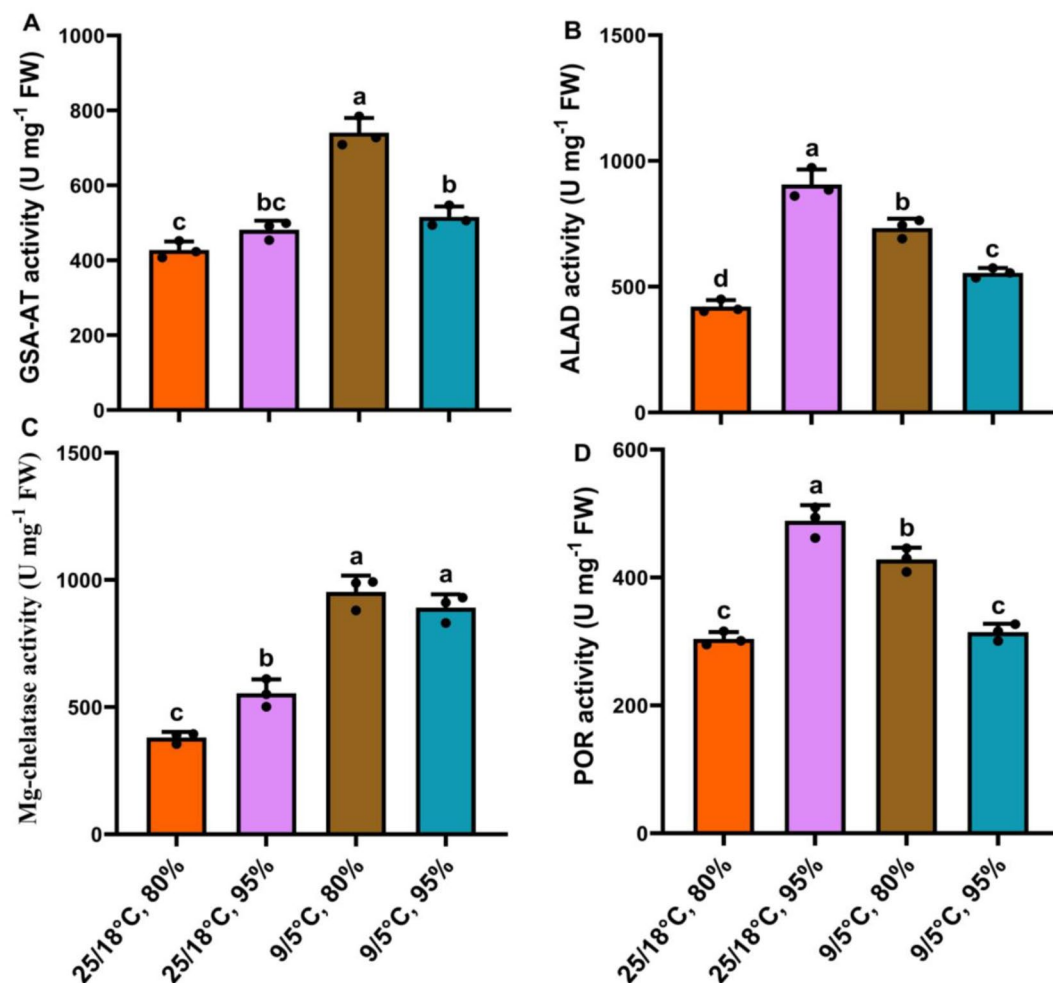
Fig. 3 Effect of low-temperature and high relative humidity on chlorophyll biosynthesis intermediates. Soluble protein (**A**), δ-Amino levulinic acid (ALA, **B**), porphobilinogen (PBG, **C**), uroporphyrinogen III (urogen III, **D**), coproporphyrinogen III (coprogen III, **E**), protoporphyrin IX (Proto IX, **F**), Mg-protoporphyrin IX (Mg-proto IX, **G**), Mg-protoporphyrin monomethyl ester (Mpe, **H**), and protochlorophyllide (Pchlide, **I**) content of cucumber seedling treated with low-temperature and high relative humidity. CK: 25/18℃, 80% represent control condition; CK+HRH: 25/18℃, 95% represent control temperature and high relative humidity; LT: 9/5℃, 80% represent low-temperature and control humidity; LT+HRH: 9/5℃, 95% represent low-temperature and high relative humidity combination. Values are means±SD from three biological replicates, ANOVA and Tukey HSD test, $P < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment

Effect of LT and HRH on the content of Phenols, flavonoids and Lignin

The content of total phenols, total flavonoids, and lignin increased significantly in all treated cucumber seedlings compared to the control plants (Fig. 7). The highest content of total phenolics and lignin was observed under LT (Fig. 7A and C), while a higher concentration of flavonoids was detected under CK+HRH (Fig. 7B). Our results reveal that individual and combined stresses of low-temperature and high relative humidity led to a surge in the concentration of total phenolics, total flavonoids, and lignin in cucumber seedlings.

Effect of LT and HRH stress on phenolic acids

Analysis of the results revealed a significant increase in phenolic acids under all three stress conditions compared to the control (Fig. 8). Our results demonstrated that the CK+HRH, LT and LT+HRH significantly increased the level of cinnamic acid than the control (Fig. 8A), and LT and LT+HRH increased the caffeic acid content (Fig. 8B) compared to the control group. The P-coumaric acid (Fig. 8C) and ferulic acid (Fig. 8D) content were increased under all the three conditions (maximum levels in LT) compared to the control. According to our results combination of low-temperature and humidity stress increases phenolic acids content in cucumber seedlings.

Fig. 4 Effect of low-temperature and high relative humidity on chlorophyll biosynthesis enzyme activities. Glutamate-1-semialdehyde transaminase (GSA-AT, **A**), ALA dehydratase (ALAD, **B**), Magnesium chelatase, (Mg- chelatase, **C**), protochlorophyllide oxidoreductase (POR, **D**) activities in cucumber seedlings under low-temperature and high relative humidity. CK: 25/18℃, 80% represent control condition; CK + HRH: 25/18℃, 95% represent control and high relative humidity; LT: 9/5℃, 80% represent low-temperature and control humidity; LT + HRH: 9/5℃, 95% represent low-temperature and high relative humidity combination. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, *P* < 0.05. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment

Effect of LT and HRH on ROS accumulation by DAB and NBT staining

Next, the indicators of oxidative damage against low-temperature and high relative humidity stress were determined (Fig. 9A and B) in the leaves of plants subjected to different treatments and levels of superoxide anion radical ($O_2^{.-}$), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA) were quantified (Fig. 9, C, D, E). Histochemical examination and measurement showed that $O_2^{.-}$ and H_2O_2 accumulation slowly accelerated in each treatment compared to control. A pronounced ROS surge was observed in plants subjected to concurrent LT + HH (Fig. 9A, B). In the control plants, a lower MDA content was observed, whereas the plant under LT and LT + HH conditions exhibited a significantly elevated MDA content (Fig. 9E), indicating that these stresses lead to higher lipid peroxidation and disruption of membrane integrity.

Discussion

Cucumber cultivation has economic importance in several countries, including China, with a growing preference for high tunnels to meet market demands [34]. However, in northern regions, the concurrent occurrence of LT and HRH poses a considerable challenge to its cultivation and productivity [2, 6]. The impact of LT + HRH stress on cucumber seedlings growth and chlorophyll formation is a critical aspect of plant morpho-physiology with significant ecological and agricultural implications [38]. LT is a primary factor with a detrimental impacting on plant growth and biomass [37]. In our study, these morphometric indicator of cucumber transplants were significantly impeded by the LT + HRH (Fig. 1A; Table 1). The growth and biomass alterations impact the overall plant structure, which, in turn, affects the distribution and density of chlorophyll in leaves [37]. Reduced
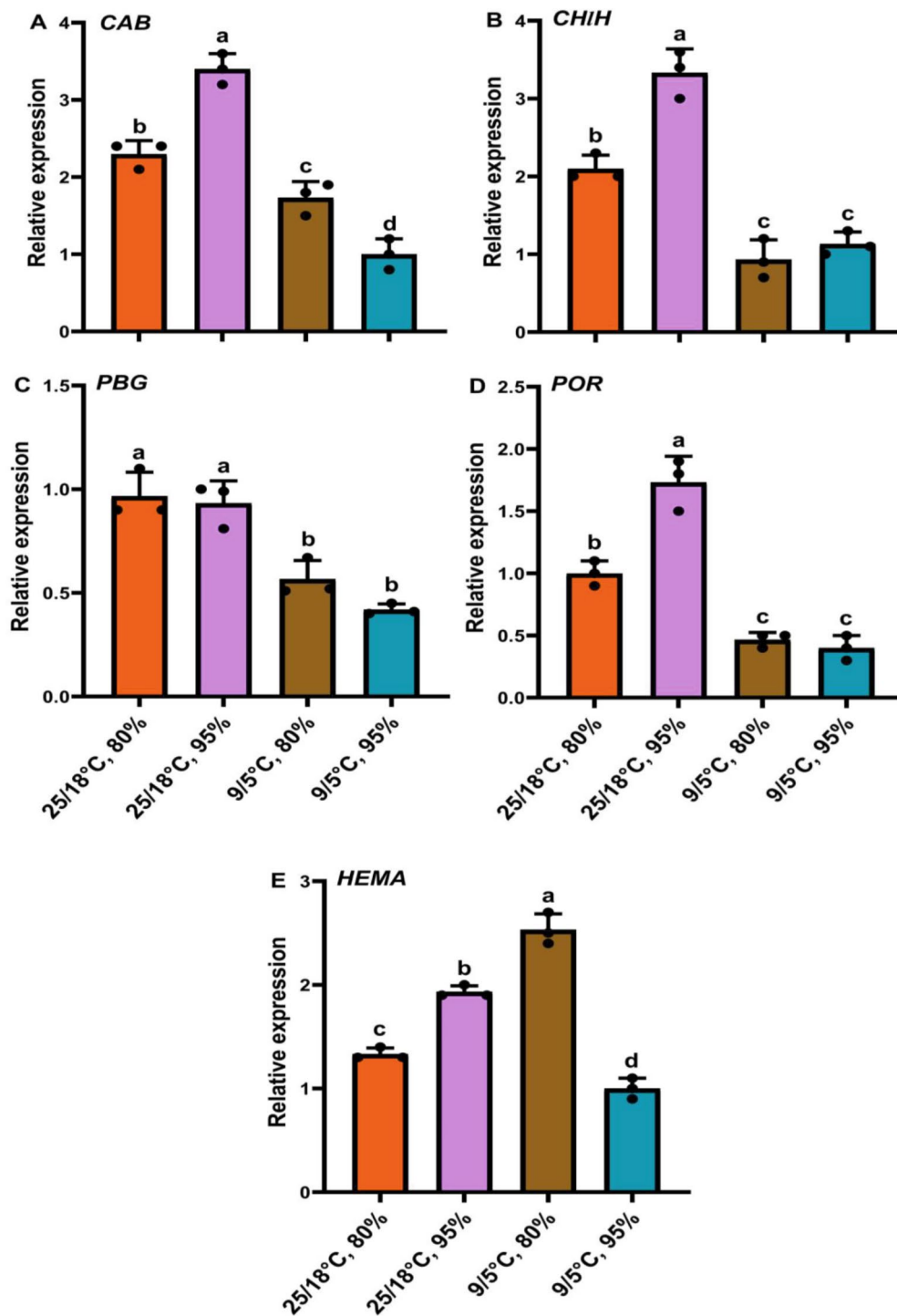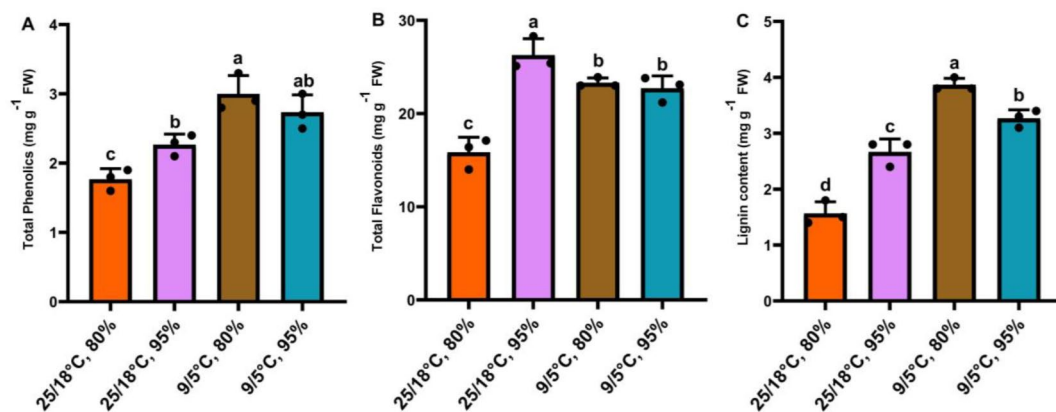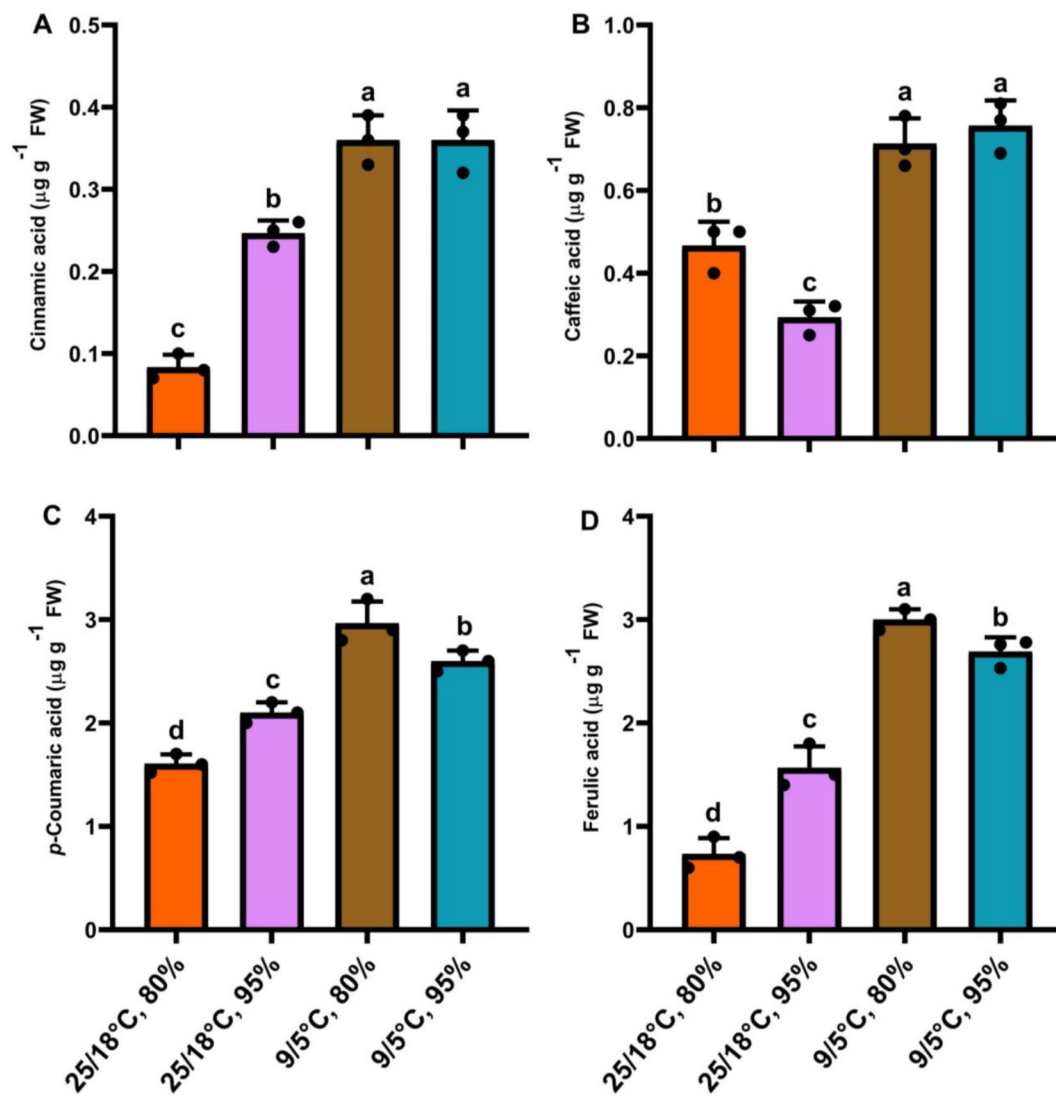
Fig. 5 Effect of low-temperature and high relative humidity on relative expression of chlorophyll biosynthesis genes, CAB (**A**), CHIH (**B**), PBG (**C**), POR (**D**) and Hema (**E**). CK: 25/18℃, 80% represent control condition; CK + HRH: 25/18℃, 95% represent control temperature and high relative humidity; LT: 9/5℃, 80% represent low-temperature and control humidity; LT + HRH: 9/5℃, 95% represent low-temperature and high relative humidity combination. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $P < 0.05$. Different letters on the bars represent significant differences and same letters represent non-significant difference among treatment
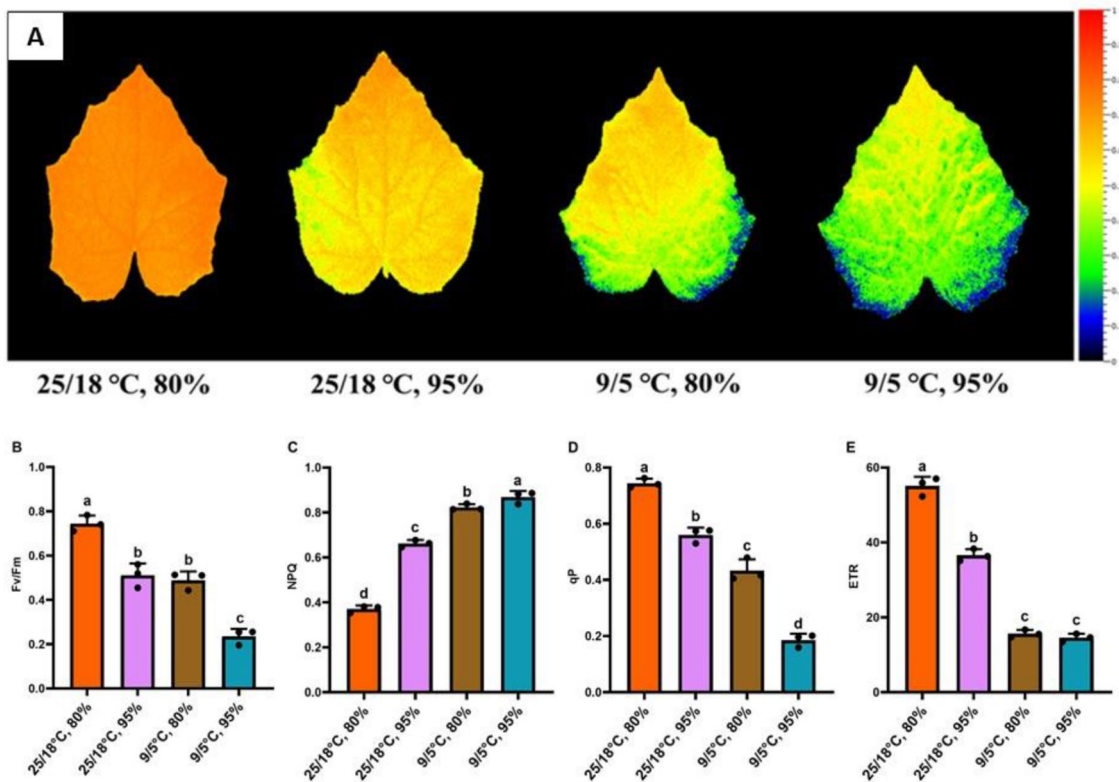
Fig. 6 Effect of low-temperature and high relative humidity on chlorophyll fluorescence parameters of cucumber seedling. The chlorophyll fluorescence images (**A**), and chlorophyll fluorescence parameters, PSII maximum efficiency (F_v/F_m, **B**), non-photochemical quenching (NPQ, **C**), photochemical quenching (qP, **D**), and electron transport rate (ETR, **E**) were detected of cucumber seedlings subjected to low-temperature and high relative humidity. CK: 25/18℃, 80% represent control condition; CK + HRH: 25/18℃, 95% represent control temperature and high relative humidity; LT: 9/5℃, 80% represent low-temperature and control humidity; LT + HRH: 9/5℃, 95% represent low-temperature and high relative humidity combination. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $P < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment

Fig. 7 Effect of low-temperature and high relative humidity on the content of total phenols (**A**), total flavonoids (**B**) and lignin content (**C**) in cucumber seedling. CK: 25/18℃, 80% represent control condition; CK + HRH: 25/18℃, 95% represent control temperature and high relative humidity; LT: 9/5℃, 80% represent low-temperature and control humidity; LT + HRH: 9/5℃, 95% represent low-temperature and high relative humidity combination. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $P < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment
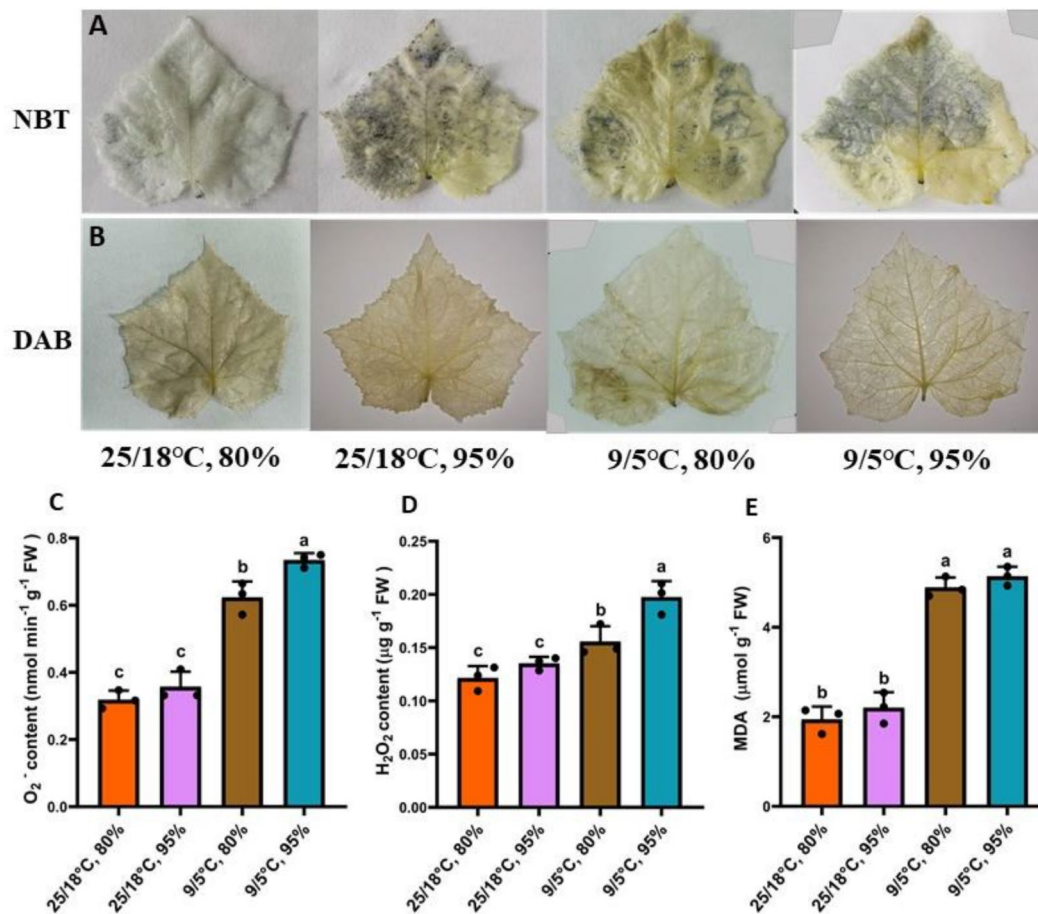
Fig. 8 Effect of low-temperature and high relative humidity on free phenolic acids, cinnamic acid (**A**), Caffeic acid (**B**), *p*-coumaric acid (**C**), and Ferulic acid (**D**) in cucumber leaves. CK: 25/18℃, 80% represent control condition; CK+HRH: 25/18℃, 95% represent control temperature and high relative humidity; LT: 9/5℃, 80% represent low-temperature and control humidity; LT+HRH: 9/5℃, 95% represent low-temperature and high relative humidity combination. Values are means±SD from three biological replicates, ANOVA and Tukey HSD test, *P* < 0.05. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment

chlorophyll is observed under LT and HRH stress due to the inhibition of chlorophyll biosynthesis and increased chlorophyll degradation, both of which are influenced by changes in cucumber seedling morphology (Fig. 2). Chlorophyll content indicates the physiological condition of plants and is essential for photosynthetic reactions, whereas carotenoid protects the photosynthetic system [38]. The chlorophyll biosynthesis is influenced by a variety of environmental stresses. Previous report stated that water and salt stress restrict chlorophyll production severely during de-etiolation [37, 38]. Fluctuation in temperature adversely affects chlorophyll biosynthesis [36]. Leaf chlorophyll pigments are affected by two factors, genetic and environmental, which alter pigment ratio (i.e., Chl and Carotenoid), leading to the different leaf colors [41]. Chlorophyll pigments are essential for capturing light energy during photosynthesis, but their function can be significantly impaired by LT, leading to reduced photosynthetic efficiency [42]. In this study the reduction of ALA indicates that LT+HRH hindered chlorophyll biosynthesis, resulting in a considerable decrease in chlorophyll pigments. Similar alterations have been observed in wheat and rice seedling subjected to sodium and water logged-stress treatments [37, 38].

LT and HRH stress interfere entrance of direct sunlight on leaves, which could lead to a decrease in photosynthetic pigments [34]. Proto IX, Mg-proto IX and Chlide contents were considerably lowered in treated plants

Fig. 9 Effect of low-temperature and high relative humidity on cucumber seedling O_2^-, H_2O_2 content. Staining of nitro blue tetrazolium (NBT, **A**), 3,3'-Di-aminobenzidine images (DAB, **B**), O_2^- content (**C**), H_2O_2 content (**D**) and MDA (**E**) under low-temperature and high relative humidity. CK: 25/18℃, 80% represent control condition; CK + HRH: 25/18℃, 95% represent control temperature and high relative humidity; LT: 9/5℃, 80% represent low-temperature and control humidity; LT + HRH: 9/5℃, 95% represent low-temperature and high relative humidity combination. Values are means ± SD from three biological replicates, ANOVA and Tukey HSD test, $P < 0.05$. Different letters on the bars represent significant differences while same letters represent non-significant difference among treatment

throughout the chlorophyll synthesis process, which were in line with previous results stated that water stress reduced Mg-chelatase and POR activities in rice seedlings [40]. The thylakoid components of the photosynthetic machinery play a critical role in modulating responses to temperature-induced stress [43]. Pchlide (Protochlorophyllide) is an essential chlorophyll precursor in plants. Protoporphyrin (Proto IX), magnesium protoporphyrin (Mg-proto IX), and Pchlide are collectively referred to as porphyrins, play a vital role in the chlorophyll biosynthesis pathway [44]. *CHLH* is the primary subunit responsible for magnesium chelation in chlorophyll biosynthesis, converting protoporphyrin IX into Mg-protoporphyrin IX [44]. Mutations in genes encoding *CHLD* and *CHLI* subunits can reduce Mg-chelatase activity, thereby inhibiting chlorophyll synthesis [17]. In our study, the *ChlH* gene was upregulated under CK + HRH conditions. However, the *ChlH* expression was significantly

downregulated under LT and LT + HRH, possibly to mitigate oxidative stress from excess tetrapyrrole intermediates, which suggesting that LT + HRH stress may suppress *ChlH* expression, potentially linked to reduced histone acetylation. Post-translational regulation plays a critical role in chlorophyll biosynthesis [39]. In Arabidopsis thaliana, there are three types of POR isoenzymes: PORA, PORB, and PORC. PORA transcripts build up in seedlings grown in the dark but decrease sharply when exposed to light. PORB is present throughout the plant's life, while PORC expression is increased by light and mainly found in mature green tissues [21]. In our study, POR expression was notably reduced in cucumber seedlings exposed to LT and LT + HRH conditions.

Increased levels of phenolic compounds were detected in cucumber seedling subjected to LT and HRH stresses, which were in lined with previous research [15] showing a correlation between plant stress responses and increase

phenolic compound levels. Increased soluble phenolic, flavonoid and lignin concentrations have also been reported in *L. esculentum* and *C. vulgaris* against cold environment, attributed to enhanced activity of phenylalanine ammonia-lyase, which catalyzes the first step of the phenolic biosynthesis pathway by converting L-phenylalanine to trans-cinnamic acid [46].

Elevated levels of reactive oxygen species (ROS) may result in lipid peroxidation, causing harm to cells and ultimately leading to cell demise [45]. Cold stress exacerbates ROS levels, thereby affecting the membrane's flexibility and the osmotic balance of plant cells [46]. The excessive generation ROS, like O_2^- and H_2O_2 triggers oxidative stress, leading to damage to the membrane structure and macromolecules within plants [42]. In the present study, a Histochemical analysis of DAB and NBT staining was done on cucumber transplants. This analysis aimed to identify the presence of ROS induced by the combination of LT+HRH. This investigation insight our understanding of oxidative stress response and potential damage caused by these environmental factors on cucumber plants.

Conclusion

LT and HRH stresses significantly impaired cucumber seedling growth, leading to enhanced ROS production and lipid peroxidation. Our results showed the detailed regulation of photsynthetic pigment in cucumber seedling under LT, HRH and LT+HRH stress condition. Chlorphyll a, b, total chlorphyll and carotenoid levels were drastically reduced under LT+HH stress. The activities of chlorophyll biosynthetic enzymes (GSA-AT, ALAD, Mg-chelatase, POR) were increased, with maximum were observed under LT. The chlorophyll biosyntehsis gene expresion were upregulated under CK+HRH and down regulated under LT+HRH condition. Additionally, Total phenol, Total flavonoid, lignin and phenolic acids (cinnamic acid, caffeic acid, *p*-coumaric acid, ferulic acid) contents were boosted under all stress condition. These findings suggest that chlorophyll biosynthesis inhibition may result from both blocked ALA synthesis and impaired Pchlide-to-chlorophyll conversion We propose that exogenous application of ALA could increase chlorophyll biosynthesis and improve resistance to LT+HRH in cucumber seedlings.

Abbreviations

LT	low tempertaure
HH	high humidity
ALA	δ-Aminolevulinic acid
POR	protochlorophyllide oxidoreductase
POR	Protochlorophyllide oxidoreductase
PBG	Porphobilinogen
Chl	Chlorophyll
Proto IX	Protoporphyrin IX
urogen III	Uroporphyrinogen III
coprogen III	Coproporphyrinogen III
RWC	Relative Water Content
GSA-AT	Glutamate 1-semialdehyde aminotransferase
ALAD	ALA dehydratase
HPLC-MS	High Performance Liquid Chromatography-Mass Spectrometry
NaOH	Sodium hydroxide
O_2^-	Oxygen anion
H_2O_2	Hydrogen peroxide
NPQ	non-photochemical quenching coefficients
qP	photochemical quenching coefficient
ETR	electron transport rate
Car	carotenoid

Data availability

Raw data will be provided on reasonable request by corresponding author Zhihui Cheng.

Declarations

Ethics approval and consent to participate

The experimental material and reagents were provided by Zhongming Fang and Zhihui Cheng. The experiment was conducted at Northwest A&F University and Guizhou University, China.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Author details

[1]Institute of Rice Industry Technology Research, Key Laboratory of Functional Agriculture of Guizhou Provincial Department of Education, Key Laboratory of Molecular Breeding for Grain and Oil Crops in Guizhou Province, College of Agricultural Sciences, Guizhou University, Guiyang, Guizhou 550025, China
[2]Key Laboratory of Plant Resource Conservation and Germplasm Innovation in Mountainous Region (Ministry of Education), Guizhou University, Guiyang, Guizhou 550025, China
[3]College of Horticulture, Northwest A&F University, Yangling, China
[4]College of Horticulture and Landscape Architecture, Yangzhou University, Yangzhou 225009, China

5Horticultural Research Institute, National Agricultural Research Centre, Islamabad 44000, Pakistan
6Department of Horticulture and Landscape Gardening, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila, Sri Lanka
7National Key Laboratory of Green Pesticide, Key Laboratory of Green Pesticide and Agricultural Bioengineering, Center for Research and Development of Fine Chemicals, Ministry of Education, Guizhou University, Guiyang 550025, China
8Department of Biology, College of Science and Humanities, Prince Sattam bin Abdul Aziz University, Alkharj 11942, Kingdom of Saudi Arabia

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