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Humic Acid Alleviates Low-Temperature Stress by Regulating Nitrogen Metabolism and Proline Synthesis in Melon (*Cucumis melo* L.) Seedlings

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Abstract: Melon is a thermophilic crop that is extremely sensitive to temperature changes. Humic acid (HA) is an eco-friendly biostimulant that enhances plants against abiotic stresses. However, the effect of HA on the cold tolerance of melon plants under low-temperature scenarios is still unclear. This study aimed to find out the effects of HA treatment on the morphological, physiological, and nitrogen metabolism of melon seedlings under low-temperature stress. HA treatment significantly enhanced plant height, stem diameter, fresh weight, dry weight, chlorophyll content (up to 33.17%), maximum photochemical efficiency (*Fv*/*Fm*), root architecture, superoxide dismutase (SOD), and catalase (CAT) activity. HA also promoted the degradation of nitrate nitrogen (NO_3^--N); the synthesis of ammonium nitrogen (NH_4^+-N), free amino acids, proline, and soluble protein; and nitrogen metabolism (NR, GS, GOGAT, and GDH, up to 181.83%) and proline-related enzyme activity (P5CS and OAT, up to 81.97%). HA significantly increased the expression of nitrogen metabolism and proline metabolism genes. In summary, HA alleviated the damage caused by low-temperature stress by improving levels of antioxidant enzymes, nitrogen metabolism, and proline synthesis.

Keywords: humic acid; low-temperature stress; melon; nitrogen metabolism; proline synthesis

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1. Introduction

Melon (*Cucumis melo* L.), which belongs to the Cucurbitaceae family and the genus Cucumis, is widely cultivated worldwide due to its high economic value. China's melon cultivation area has reached 395,100 hectares, with a yield of 13.808 million tons, making it one of the important economic crops [1]. *Cucumis melo* L. is a thermophilic plant, and its seedlings are particularly sensitive to temperature fluctuations. Due to abnormal global climate changes, melons are susceptible to cold stress [2]. During winter and early spring in China, melon seedlings cultivated in greenhouse facilities often experience low-temperature stress, which leads to delayed harvests, reduced yields, and economic losses [3]. Low-temperature stress is one of the major abiotic stresses limiting the geographical distributions of plants, reducing crop yields [4]. When plants are subjected to cold stress, their reactive oxygen species (ROS) levels increase, which induces a series of

physiological and biochemical changes that enable plants to survive under cold stress [5]. In addition, cold stress also affects the process of nitrogen metabolism.

Nitrogen metabolism mediates plant growth, yield, and quality traits and plays a significant role in maintaining adaptive homeostasis under stress conditions [6]. Low-temperature stress inhibits crop growth by reducing the activity of key enzymes involved in nitrogen metabolism. Previous research has demonstrated that high nitrogen levels in tomato leaves are positively correlated with photosynthetic capacity and the activities of nitrate reductase (NR), glutamine synthetase (GS), and glutamate synthase (GOGAT) but negatively correlated with the activity of glutamate dehydrogenase (GDH) under low-temperature stress [7]. Low temperatures inhibit the growth of crops but increase the activities of sucrose synthase and glutamine synthetase, thereby promoting carbon and nitrogen transport [8]. Free amino acids play a crucial role in cold adaptation, as their accumulation can prevent cells from losing water under low-temperature conditions [2]. Proline triggers or participates in multiple plant cell signaling pathways involved in cellular defense, including the maintenance of osmotic homeostasis, regulation of the cellular redox status, and ROS scavenging [9]. In higher plants, proline is derived from glutamic acid (Glu) metabolism and ornithine (Orn) metabolism [10]. Stress primarily accelerates proline synthesis by promoting the activity of Δ1-pyrroline-5-carboxylate synthetase (P5CS) and Δ1-pyrroline-5-carboxylate reductase (P5CR) and inhibits the activity of proline dehydrogenase (ProDH) and Δ1-pyrroline-5-carboxylate dehydrogenase (P5CDH) [11]. The Orn pathway is generally only activated under conditions of high nitrogen availability or other types of mild stress [12]. Both glutamic acid and ornithine pathways are involved in cold-induced proline synthesis, while freezing treatment significantly up-regulates the expression of the OAT gene without significantly affecting P5CS activity [13]. Therefore, it is necessary to research methods on plant resistance to low-temperature stress.

Humic acid (HA) is an eco-friendly biostimulant that is widespread in the environment. It plays a key role in regulating soil fertility and plant nutrient uptake and has been shown to enhance the heat tolerance of *Arabidopsis thaliana* by promoting the transcriptional activity of heat shock proteins [14,15]. Seeds and seedlings treated with HA are highly resistant to various types of abiotic stress, such as salinity, drought, and heavy metal toxicity [16–18]. Humic substances (HSs) also interfere with secondary metabolism by altering gene expression and the content of compounds in plant cells and are involved in the Calvin cycle, nitrate and phosphorus metabolism, glycolysis, and photosynthesis [19]. HA enhances potato growth, photosynthetic parameters, and fresh tuber yield under various water-deficit conditions [18]; reduces Na^+ accumulation; increases K^+ accumulation; decreases leaf necrosis area and lipid peroxidation; and enhances antioxidant enzyme activity and the salt tolerance index [17]. However, there are few studies on the alleviation of low-temperature stress on melon growth inhibition through enhancing nitrogen metabolism and proline synthesis by HA.

In this study, the effects of HA on the growth, antioxidant enzyme system, nitrogen metabolism, and proline synthesis of melon seedlings under low-temperature stress were investigated through root irrigation treatment. The findings are helpful in enriching the research of plant cold resistance and deepening our understanding of HA application in agricultural production.

2. Materials and Methods

2.1. Plant Materials and Experimental Design

All the experiments were performed at Hebei Agricultural University (38°23′ N, 115°28′ E), Baoding, Hebei, China. The seeds of the melon variety "Yugu" were provided by Nongyou Seed Co., Ltd., Xiamen, Fujian, China. All the seeds were soaked in distilled

water for 6 h and then covered by a moist towel and germinated in the dark at 28 °C for 24 h. Uniformly germinated seeds were sown in 50-well trays filled with growth material containing peat, vermiculite, and perlite (2:1:1, *v*/*v*/*v*) in an artificial light climate chamber. The growth conditions were as follows: 12 h/12 h photoperiod, photosynthetic photon flux density, 300 μmol m^{-2} s^{-1}, temperature, 28 °C/18 °C (day/night), and relative humidity, 60–70%. Seedlings at the first true leaf stage were transplanted into plastic nutrient pot weight (10 cm × 12 cm) and cultivated in the same conditions till the two-leaf stage.

Prior to cold stress, two treatments were included, each containing 60 seedlings (120 seedlings total): (1) The control group (CK): Seedlings were cultured normally with deionized water. (2) The HA treatment (HA): Seedlings were cultivated with 300 mg L^{-1} HA solution (1415-93-6, Sigma-Aldrich Co., Ltd., Saint Louis, MO, USA). Seedlings were treated twice, three days apart between each treatment, and 25 mL was applied per plant. The seedlings were placed into an artificial light climate chamber for low-temperature treatment after root-irrigation treatments. The day and night temperature were 10 °C/5 °C (12 h/12 h), and the intensity of light was 300 μmol m^{-2} s^{-1}. During the cold stress, samples were collected on days 1, 3, 5, and 7 (simulated low-temperature days in early spring). The plant height, stem diameter, shoot and root fresh weight, root architecture, chlorophyll content, and chlorophyll fluorescence indexes were determined using fresh samples. The remaining plant samples were stored at −80 °C.

2.2. Growth Index Measurement and Root Architecture

Plant height and stem diameter were determined using a ruler and vernier calipers. The fresh weight was measured by using a balance. Then, samples were dried at 80 °C for constant weight to measure dry weight [20]. The roots were spread evenly on the Wanshen LA-S plant root analyzer, and images were captured and imported into the image analysis system (Hangzhou Wanshen Testing Technology Co., Ltd., Hangzhou, China) to analyze root length, diameter, volume, surface area, tips, and forks.

2.3. Total Chlorophyll Content, Fv/Fm, REL, MDA, and Antioxidant Enzyme Activity Measurement

The total chlorophyll content was measured by 80% acetone extraction method [21]. The third leaf at the top of the plant to be tested was selected and darkened for 20 min in advance, and then the *Fv*/*Fm* value of melon leaves was determined by a portable chlorophyll fluorescence analyzer (Pocket PEA plus version 1.0, Hansatech Instruments Ltd., Kings Lynn, UK). The REL was determined by Cao et al. [22].

The leaves were treated with thiobarbituric acid, and the MDA content was quantified as previously described [23]. The superoxide dismutase (SOD) and catalase (CAT) activities were determined following the method described in a previous study [24]. The peroxidase (POD) activity was measured following Chakraborty et al. [25].

2.4. NO_3^--N and NH_4^+-N Content Measurement

The NO_3^--N content was determined using the nitro-salicylic acid method [26]. The reaction mixture was composed of 0.1 mL filtrate and 0.4 mL 5% salicylic acid (dissolved in concentrated H_2SO_4), and the absorbance was recorded at 410 nm following the addition of 9.5 mL 8% NaOH. The NH_4^+-N content was determined using the ninhydrin method [27].

2.5. Proline, Soluble Protein, and Free Amino Acid Content Measurement

The proline content was determined using the sulfosalicylic acid method [28]. The absorbance was measured at 520 nm [29]. The soluble protein content was determined using the Coomassie Brilliant Blue G-250 reagent method [30]. Amino acids were extracted with acetic acid/sodium acetate buffer (pH 5.4) and measured photometrically at 580 nm [31].

2.6. Nitrogen Metabolism Enzyme Activity Measurement

About 0.1 g of the sample was weighed, and 1.0 mL of extraction solution was added and then homogenized on ice. The mixture was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was taken and then placed on ice for further measurement. According to the instructions in the kit, the enzyme activities of NR, GOGAT, and GDH were measured at a wavelength of 340 nm, while the enzyme activity of GS was measured at a wavelength of 540 nm. The activities of NR, GS, GOGAT, and GDH were determined using relevant kits (Comin Biotechnology Co., Suzhou, China).

2.7. Proline Metabolism Enzyme Activity Measurement

About 0.1 g of the sample was weighed, and then, 1.0 mL of extraction solution A and also 10 μL of extraction solution B were added. After homogenizing on ice, the mixture was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was taken and then placed on ice, pending further determination. According to the instructions in the kit, the enzyme activities of P5CS and OAT were measured at a wavelength of 340 nm, while the enzyme activities of P5CR and ProDH were measured at the wavelength of 450 nm and 600 nm. The P5CS, OAT, ProDH, and P5CR activities were determined using relevant kits (Beijing Solarbio Technology Co., Ltd., Beijing, China).

2.8. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from leaf samples using the RNA prep Pure Plant Plus Kit (Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The cDNA was reversed-transcribed using a SimpliAmp PCR system for single-strand cDNA synthesis (TransGen Biotech Co., Ltd., Beijing, China). The relative expression of genes involved in nitrogen metabolism and proline synthesis was performed in the CFX Connect Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) using the iQ SYBR Green Supermix (Bio-Rad) in a final volume of 20 μL according to the manufacturer's instructions. The primers for these genes are listed in Table 1. The amplification conditions were as follows: 94 °C for 30 s, followed by 40 cycles at 94 °C for 5 s, 58 °C for 15 s, and 72 °C for 10 s. The primers for these genes are listed in Table 1. All the measurements for samples were performed using three biological replicates. The relative expression level was calculated using the $2^{-\Delta\Delta CT}$ method.

Table 1. Sequences of primers used in qRT-PCR.

Gene	Forward (5′-3′)	Reverse (5′-3′)
NR	CAACTCAACTCACGGAGCCT	GCACATCGTGTGAGATTGCG
GS	TGGCCTTCGTTACCACCTTC	CTTCCGAAAGCGATTGAGGC
GOGAT	TGGCCTTCGTTACCACCTTC	CTTCCGAAAGCGATTGAGGC
GDH	GCTGCAACCCAAGGGAGTTA	GTCTGTGCATTTGTGCCCAT
P5CS	GCATGGAAGTGCACACACTG	AGCACCCAGACCAAATCGAG
OAT	TCCCTTGTTGCCTGGACATC	TAACCCCTGCCTCTCCTTGA
P5CR	AGTTGCTGCAGGTCTACCAC	TGGTAGTCCCACCAGGTGAT
ProDH	ATATGCCGATGACGAAGCGT	AACTCGCAGAACCAGATGGG
β-actin	GGCAGTGGTGGTGAACATG	TTCTGGTGATGGTGTGAGTC

Note: *NR*, *nitrate reductase* gene; *GS*, *glutamine synthetase* gene; *GOGAT*, *glutamate synthase* gene; *GDH*, *glutamate dehydrogenase* gene; *P5CS*, *pyrroline-5-carboxylic acid synthetase* gene; *OAT*, *ornithine transaminase* gene; *P5CR*, *pyrroline-5-carboxylate reductase* gene; *ProDH*, *proline dehydrogenase* gene; *β-actin: internal standard.*

2.9. Statistical Analysis

The experiment was conducted in a fully randomized design with three replicates. *t*-tests in SPSS v. 22.0 (SPSS Inc., Chicago, IL, USA) were used to analyze the data. * $p < 0.05$

was considered significant, and ** $p < 0.01$ was considered extremely significant. The processed data were plotted using GraphPad Prism (version 8.0).

3. Results

3.1. Effects of HA on Growth Parameters, the Chlorophyll Content, and Fv/Fm Under Cold Stress

Both CK- and HA-treated plants were subjected to 1 day of low-temperature treatment, and no significant differences in their growth were observed. Mild wilting was observed in CK plants after 3 days, and HA-treated plants continued to grow normally. After 5 days, CK plants showed significant wilting, and HA seedlings experienced only mild wilting. Both the CK and HA treatments exhibited noticeable wilting after 7 days (Figure 1A).

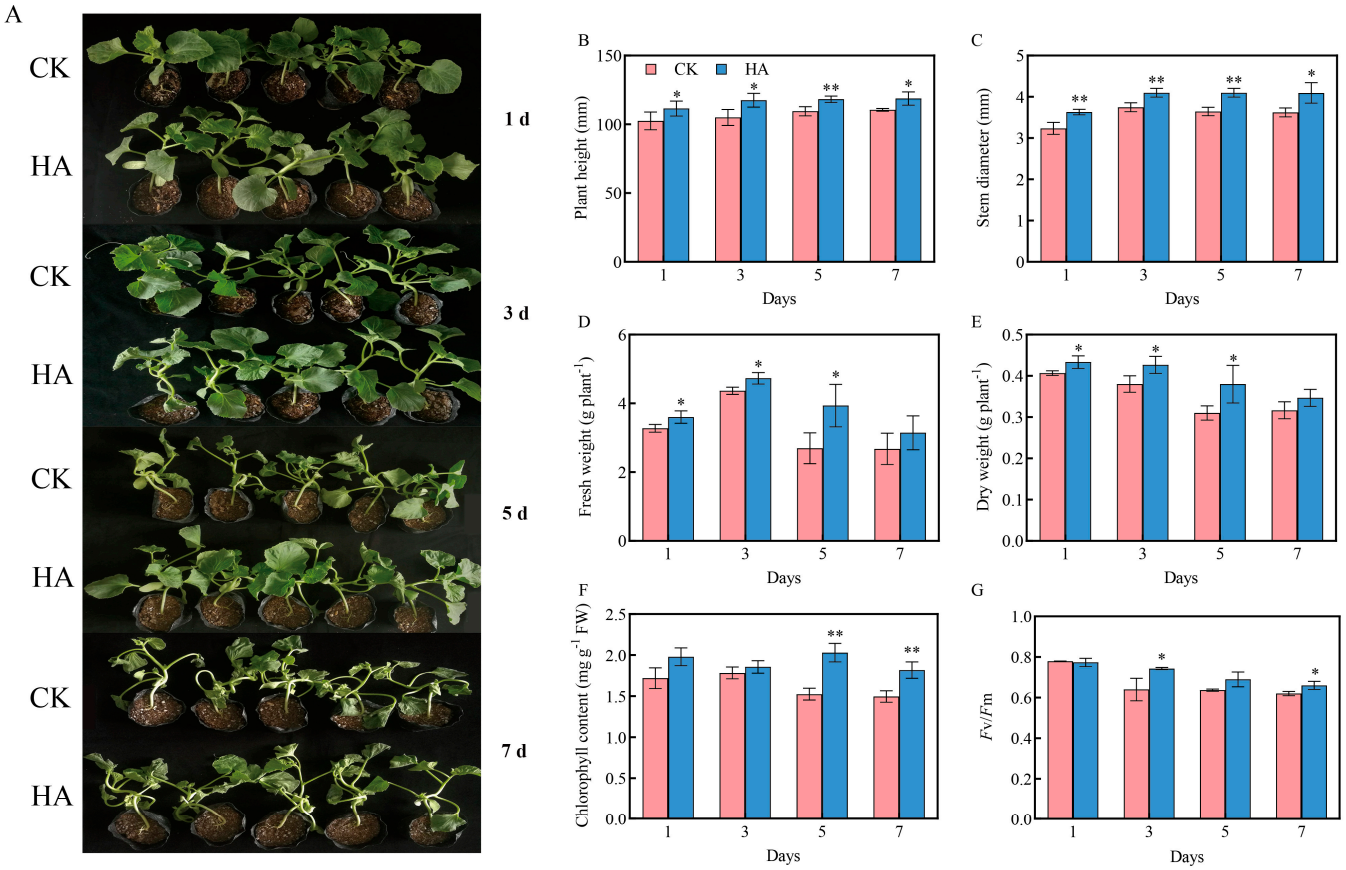

Figure 1. The effects of HA on growth parameters, the chlorophyll content, and *Fv*/*Fm* under cold stress: Phenotype (**A**), plant height (**B**), stem diameter (**C**), fresh weight (**D**), dry weight (**E**), chlorophyll content (**F**), and *Fv*/*Fm* (**G**) of melon seedlings under cold stress. An independent *t*-test was used to analyze differences between HA and control treatments. Each value is the mean of three replicates. Vertical bars indicate standard deviation (SD). * $p < 0.05$; ** $p < 0.01$.

Under low-temperature stress, the growth of control plants was slow. HA significantly increased the growth index of the plants. On days 1, 3, 5, and 7, the plant height of HA-treated seedlings was 12.08%, 12.26%, 9.19%, and 10.48% higher than that of the CK, respectively; the stem diameter was 8.76%, 5.43%, 6.63%, and 8.45% higher than that of the CK, respectively. The fresh weight was 7.83% and 12.96% higher at day 1 and day 3 of the HA treatment compared with the CK, and the dry weight was 8.33% and 5.00% higher at day 1 and day 3 of the HA treatment compared with the CK, respectively; these differences were significant (Figure 1B–E). The chlorophyll content was 33.17% and 21.50% higher at day 5 and day 7 of the HA treatment than in the CK, respectively (Figure 1F). HA treatment

significantly delayed the decrease in *Fv*/*Fm*, and the differences between the HA treatment and CK were significant at day 3 and day 7 (Figure 1G).

3.2. Effects of HA on Root Architecture Under Cold Stress

Under low-temperature stress, the root architecture of HA-treated seedlings was significantly enhanced compared with that of CK seedlings. After 3 days of treatment, the length, surface area, volume, diameter, number of tips, and number of forks of seedlings were 27.31%, 71.31%, 90.72%, 15.13%, 30.77%, and 45.02% higher in the HA treatment than in the CK, respectively (Figure 2).

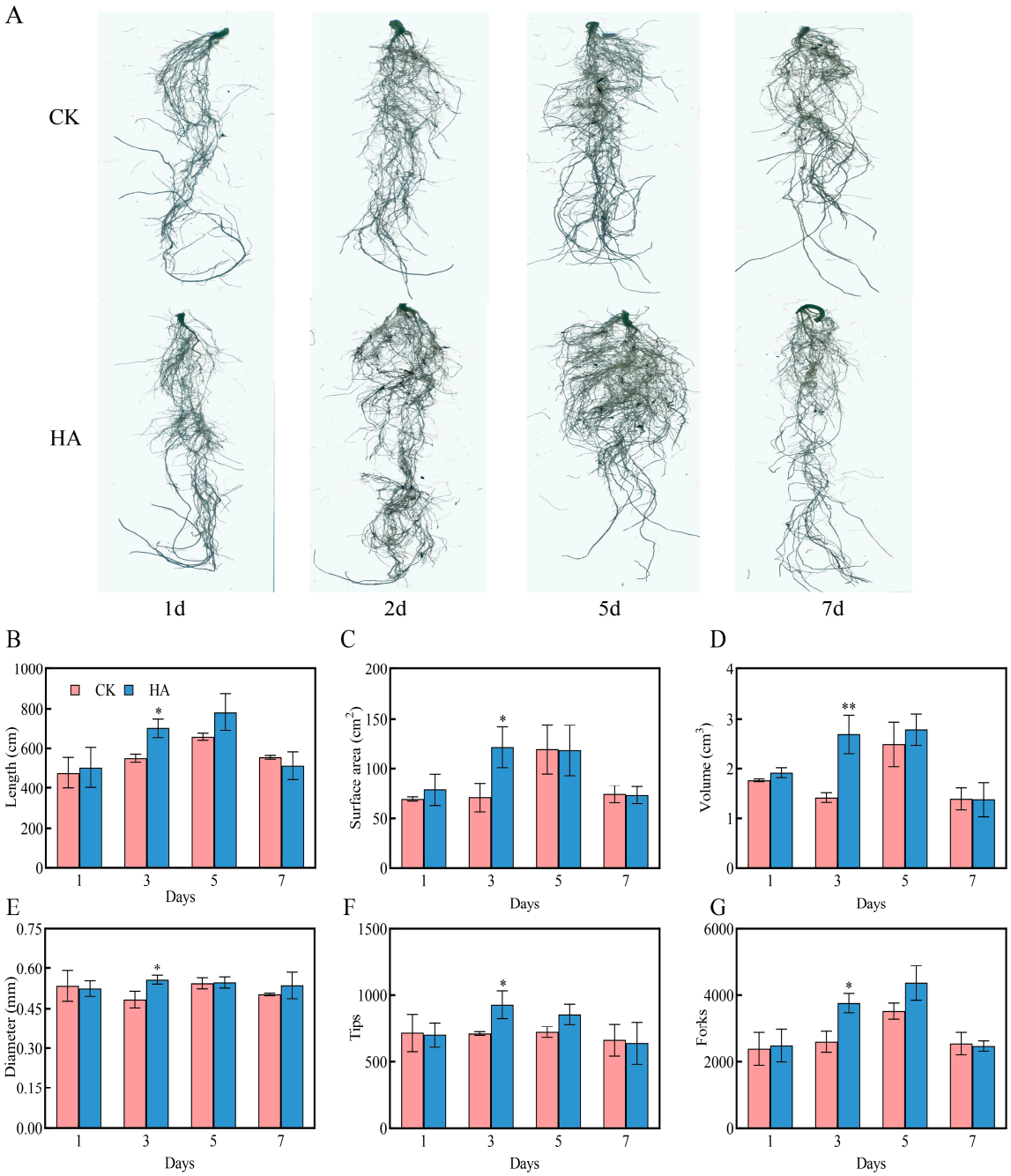

Figure 2. Effects of HA on root architecture under cold stress: phenotype (**A**), length (**B**), surface area (**C**), volume (**D**), diameter (**E**), tips (**F**), and forks (**G**). An independent *t*-test was used to analyze differences between HA and control treatments. Each value is the mean of three replicates. Vertical bars indicate standard deviation (SD). * $p < 0.05$; ** $p < 0.01$.

3.3. Effects of HA on Antioxidant Enzyme Activity, REL, and MDA Content Under Cold Stress

Under low-temperature stress, the SOD activity of both the CK and HA treatments gradually decreased, and the CAT and POD activities gradually increased. Specifically, SOD activity was 10.45%, 22.30%, 7.09%, and 75.00% higher in the HA treatment than in the CK on days 1, 3, 5, and 7, respectively. CAT activity was 26.92%, 7.84%, 6.42%, and 17.49% higher in the HA treatment than in the CK on days 1, 3, 5, and 7, respectively. POD activity was significantly lower in the HA treatment than in the CK on day 7 (Figure 3A–C). The REL and MDA content of melon seedlings in the CK and HA treatment gradually increased. At 3, 5, and 7 days, the REL was 15.89%, 7.38%, and 21.85% lower in the HA treatment than in the CK, and the MDA content was 9.38%, 11.97%, and 11.98% lower in the HA treatment than in the CK, respectively (Figure 3D,E).

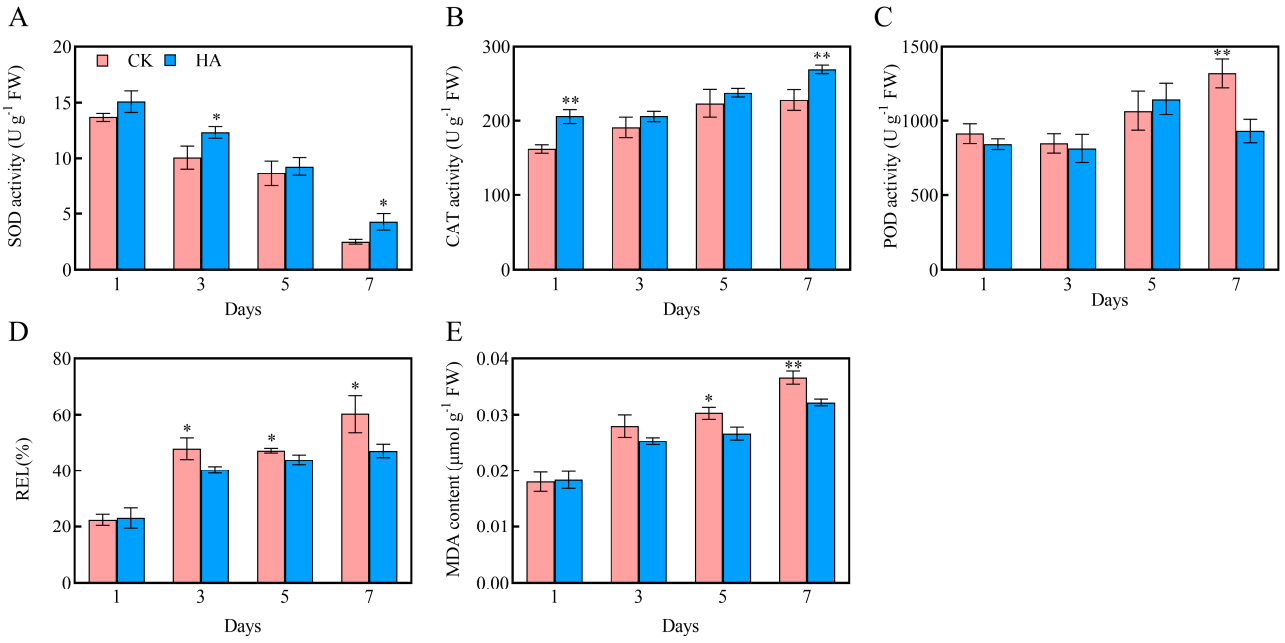

Figure 3. Effects of HA on antioxidant enzyme activity, REL, and the MDA content under cold stress: SOD activity (**A**), CAT activity (**B**), POD activity (**C**), REL (**D**), and MDA content (**E**) in HA-treated and control leaves of melon seedlings under cold stress. An independent *t*-test was used to analyze differences between HA and control treatments. Each value is the mean of three replicates. Vertical bars indicate standard deviation (SD). * $p < 0.05$; ** $p < 0.01$.

3.4. Effects of HA on the Content of NO_3^--N, NH_4^+-N, Free Amino Acids, Proline, and Soluble Protein Under Cold Stress

The NO_3^--N content was 8.43%, 21.93%, 23.51%, and 7.13% lower in the HA treatment than in the CK on days 1, 3, 5, and 7, respectively (Figure 4A). The NH_4^+-N content was 8.33% lower in the HA treatment than in the CK on day 1, and it was 68.83% and 15.33% higher in the HA treatment than in the CK on days 3 and 5 (Figure 4B).

The content of leaves' free amino acids and proline first decreased and then increased in both the CK and HA treatments. The free amino acid content was 31.73%, 8.25%, and 6.92% higher in the HA treatment than in the CK on days 3, 5, and 7, respectively. The proline content was 15.36%, 24.83%, 25.69%, and 42.68% higher in the HA treatment than in the CK on days 1, 3, 5, and 7, respectively, and these differences were significant. The soluble protein content was 17.57% and 13.61% higher in the HA treatment than in the CK on days 1 and 5, respectively, and these differences were significant (Figure 4C–E).

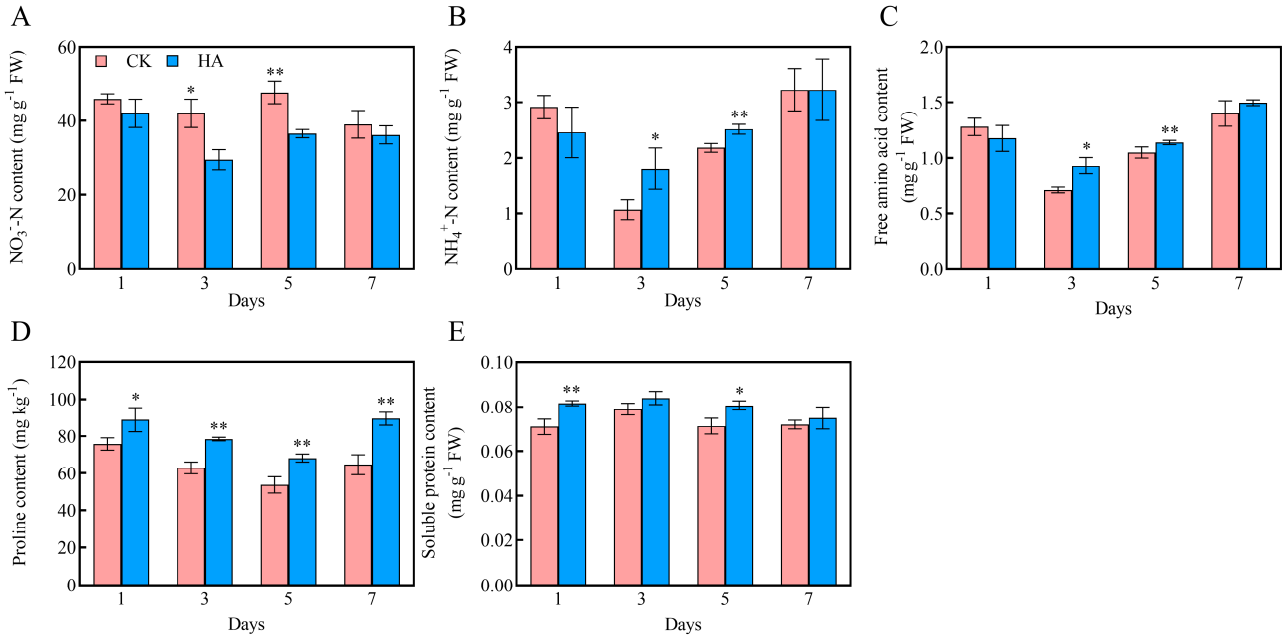

Figure 4. Effects of HA on the content of NO_3^--N, NH_4^+-N, free amino acids, proline, and soluble protein under cold stress: NO_3^--N content (**A**), NH_4^+-N content (**B**), free amino acids content (**C**), proline content (**D**), and soluble protein content (**E**). An independent *t*-test was used to analyze differences between HA and control treatments. Each value is the mean of three replicates. Vertical bars indicate standard deviation (SD). * $p < 0.05$; ** $p < 0.01$.

3.5. Effects of HA on Nitrogen Metabolism Enzyme Activity and Relative Expression Levels of Genes Under Cold Stress

Under low-temperature stress, the activities of nitrate reductase (NR), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) in the leaves of both the CK and HA treatments decreased significantly (Figure 5A–D). The average NR activity in the HA treatment was 181.83% higher than that in the CK across all time points; GS activity increased by an average of 9.21%; GOGAT activity increased by an average of 65.22%; and GDH activity increased by 26.53%.

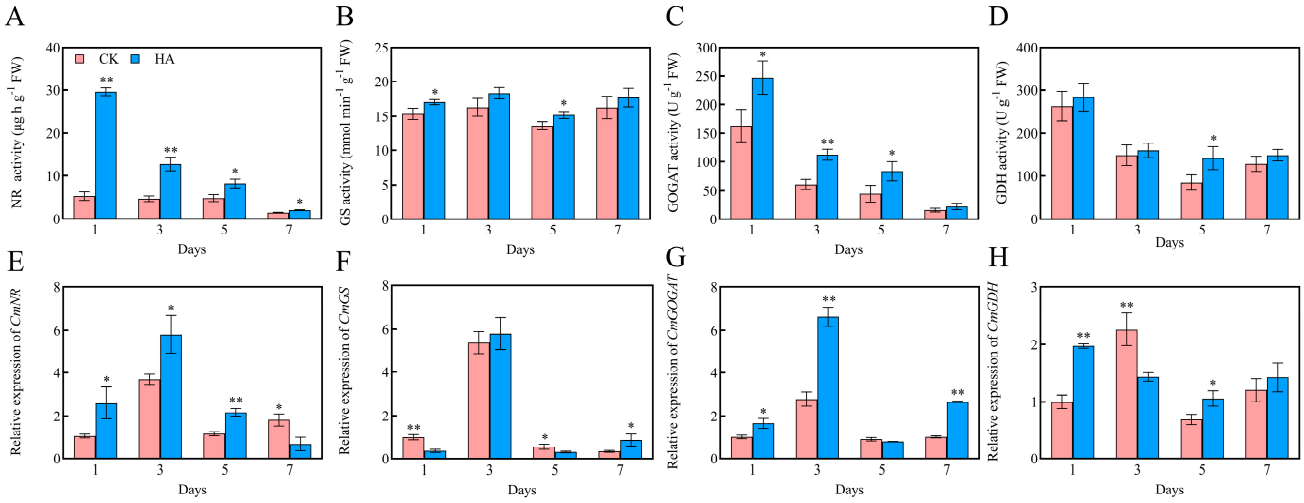

Figure 5. Effects of HA on nitrogen metabolism enzyme activity and relative expression levels of genes under cold stress: NR activity (**A**), GS activity (**B**), GOGAT activity (**C**), and GDH activity (**D**) and the relative expression of *CmNR* (**E**), *CmGS* (**F**), *CmGOGAT* (**G**), and *CmGDH* (**H**). An independent *t*-test was used to analyze differences between HA and control treatments. Each value is the mean of three replicates. Vertical bars indicate standard deviation (SD). * $p < 0.05$; ** $p < 0.01$.

Compared with the CK, the relative expression of *CmNR* in the HA treatment was significantly up-regulated by 144.99%, 57.19%, and 86.38% on days 1, 3, and 5, respectively; the relative expression of *CmGS* was up-regulated by 6.69% and 156.54% on days 3 and 7, respectively; the relative expression of *CmGOGAT* was up-regulated by 65.07%, 137.41%, and 212.91% on days 1, 3, and 7, respectively; and the relative expression of *CmGDH* was up-regulated by 94.70%, 33.17%, and 18.20% on days 1, 5, and 7, respectively (Figure 5E–H).

3.6. Effects of HA on Proline Metabolism Enzyme Activity and Relative Expression Levels of Genes Under Cold Stress

The average P5CS activity was 81.97% higher in the HA treatment than in the CK (Figure 6A). The average OAT activity was 54.04% higher in the HA treatment than in the CK (Figure 6B). P5CR activity was only 10.19% higher in the HA treatment than in the CK on day 3, and it was lower in the HA treatment than in the CK on the other days (Figure 6C). The average ProDH activity was 48.77% lower in the HA treatment than in the CK (Figure 6D).

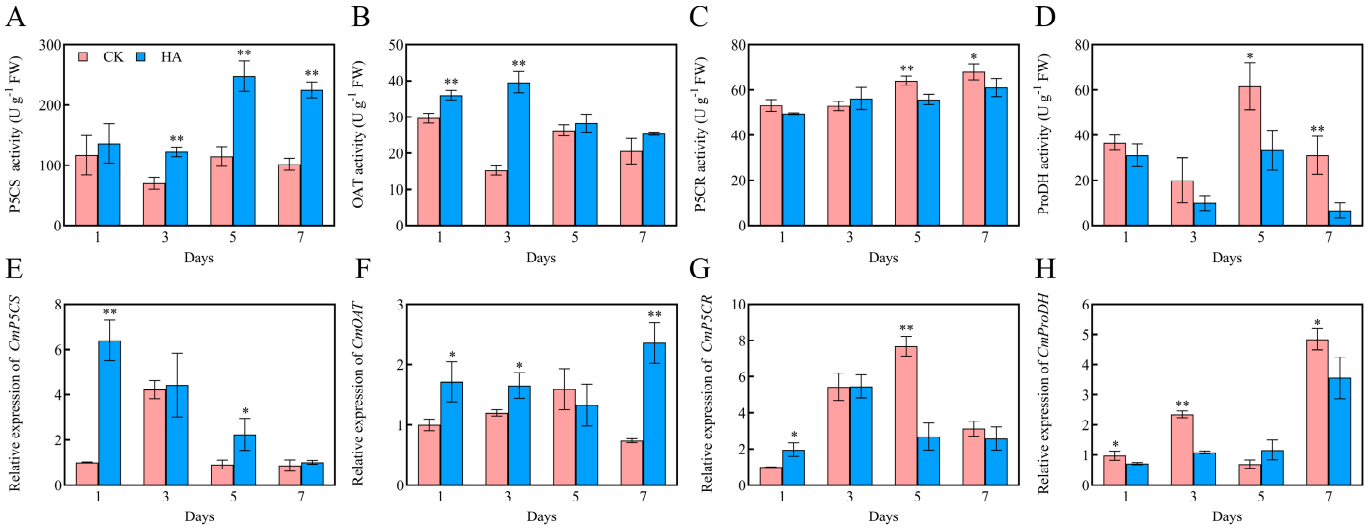

Figure 6. Effects of HA on proline metabolism enzyme activity and relative expression levels of genes under cold stress: P5CS activity (**A**), OAT activity (**B**), P5CR activity (**C**), and ProDH activity (**D**) and the relative expression of *CmP5CS* (**E**), *CmOAT* (**F**), *CmP5CR* (**G**), and *CmProDH* (**H**). An independent *t*-test was used to analyze differences between HA and control treatments. Each value is the mean of three replicates. Vertical bars indicate standard deviation (SD). * $p < 0.05$; ** $p < 0.01$.

Compared with the CK, the relative expression level of *CmP5CS* in the HA treatment was up-regulated by an average of 177.75% at various time points (Figure 6E). The relative expression level of *CmOAT* was up-regulated by an average of 105.66% on days 1, 3, and 7 (Figure 6F). The relative expression level of *CmP5CR* was up-regulated by 96.31% on day 1 but down-regulated by 65.00% and 17.47% on days 5 and 7, respectively (Figure 6G). The relative expression level of *CmProDH* was down-regulated by an average of 108.17% on days 1, 5, and 7 (Figure 6H).

4. Discussion

Low temperatures severely inhibited plant growth and led to physiological disorders [32,33]. HA can alleviate the inhibition of abiotic stress on plant growth. For example, HA can increase the leaf area and pigment content, promote the absorption of nitrogen, phosphorus, and potassium ions, fruit yield, and quality in mango and oilseed rape [34,35]. HA can also increase the total dry weight, fruit yield, and leaf area of pepper [36] and strawberries under salt stress [17]. In this study, HA treatment significantly increases the

plant height, stem diameter, fresh weight, and dry weight of melon seedlings under cold stress. HA significantly promoted root growth under low-temperature stress and optimized root morphology.

Generally, under stress, the physiological and biochemical reactions of plants are altered, and these alterations mainly affect photosynthesis, the plasma membrane, and intracellular substances. Previous studies have shown that both HA and fulvic acid can improve growth parameters, photosynthetic pigments, and antioxidant levels in *Achillea millefolium* [37]. HS from different origins improves the growth of rice plants by promoting photosynthetic capacity and nitrogen metabolism [38]. Our results indicated that HA maintains a high chlorophyll content, which significantly delays the degradation of chlorophyll under low-temperature conditions. The rapid decrease in *F*v/*F*m indicates that low temperature affects the light-capturing efficiency of antenna pigments in the leaves of melon seedlings, reducing the efficiency of light energy conversion and leading to photoinhibition. HA significantly delayed the downward trend of this index (Figure 1), which helped protect structural changes in the PSII complex under low-temperature stress. HA also increased the chlorophyll content and the openness of the PSII reaction center. By reducing mesophyll resistance, it increased the absorption and utilization of light energy.

Environmental stress, including cold stress, triggers the production of ROS, resulting in membrane lipid peroxidation. Tomato plants accumulate a large amount of ROS under low-temperature stress, causing severe damage to plants [39]. The SOD, CAT, POD, and APX are important antioxidant enzymes and ROS scavengers [40]. The application of HA can effectively increase the activities of guaiacol peroxidase (GPX) and CAT in grape leaves, reducing hydrogen peroxide (H_2O_2) and the MDA content [41]. Humus molecules can increase water retention in wheat leaves under stress conditions, activating photosynthesis and antioxidant metabolism [42]. In addition, low-temperature stress can induce the relative expression levels of P5CS and P5CR, improve the activity of antioxidant enzymes, and effectively promote the accumulation of proline in wheat and pepper [43,44]. Previous studies have shown that proline enhances the cold resistance of plants by protecting subcellular structures and protein integrity, enhancing the activity of different enzymes, and scavenging singlet oxygen in reactive oxygen species [45]. Exogenous proline can reduce MDA and H_2O_2 contents by promoting proline accumulation and antioxidant enzyme activity in the orange root system, thus alleviating the inhibition of boron stress on root growth [46]. This result showed that HA treatment significantly induced up-regulated expression of *CmP5CS* and *CmOAT* and down-regulated expression of *CmProDH*. The proline, free amino acid, and soluble protein content was significantly higher in melon seedlings with HA treatment than CK. Meanwhile, SOD and CAT activities were improved, and excessive reactive oxygen species were prevented from entering by protecting the cell membrane structure, which alleviated the growth inhibition of melon seedlings under cold stress.

Nitrogen metabolism-related enzyme activities play a crucial role in plant growth by influencing nitrogen absorption and protein synthesis [47,48]. Ammonium is the preferred nitrogen source for plants [49]. The GS/GOGAT pathway is the main route mediating the conversion of NH_4^+-N into glutamate. GDH may have a unique physiological role by mediating the release of large amounts of ammonium under stress and senescence conditions. Studies have shown that low temperature inhibits the growth of crops and increases the activities of sucrose synthase and glutamine synthetase, thereby promoting the transport of carbon and nitrogen [8]. Under natural conditions, HA promotes the absorption and assimilation of N by up-regulating the expression of genes related to NO_3^--N and NH_4^+-N [50,51]. In this study, HA significantly up-regulated the relative expression levels of *CmNR*, *CmGOGAT*, and *CmGDH* and increased the activities of NR, GS,

GOGAT, and GDH and reduced the NO_3^--N content under cold stress (Figure 5), which promoted nitrate degradation in melon leaves, indicating that HA can maintain ammonia assimilation by regulating the GS/GOGAT and GDH pathways, thereby preventing the excessive accumulation of NH_4^+-N in melon seedlings under low-temperature conditions and maintaining nitrogen metabolism homeostasis [52]. The root morphology and spatial distribution also significantly affect the nitrogen absorption efficiency of plants [53]. We found that the root architecture of HA-treated seedlings was enhanced compared with that of CK seedlings, which was consistent with the findings that HA enhanced the activity of enzymes involved in nitrogen metabolism. Based on the results of this study, it is recommended to apply 300 mg L^{-1} HA for root irrigation twice within 1 week after transplanting melon seedlings and 7–10 days before the onset of extreme low-temperature weather. However, further research is needed to explore the application of humic acid in mitigating low-temperature stress in melons in actual production.

5. Conclusions

Under low-temperature stress, melon seedlings treated with HA effectively alleviated the inhibition effects of cold stress on their growth by increasing leaf pigment content and *F*v/*F*m, decreasing MDA and REL, enhancing the antioxidant system and nitrogen metabolism enzyme activities, promoting the balance of key enzyme activities involved in proline synthesis and degradation, and influencing the expression levels of related genes. These findings provide new insights into the ability of HA to enhance the cold tolerance of melon seedlings under low-temperature conditions. It is recommended to apply 300 mg L^{-1} HA for root irrigation to melon seedlings twice. Further study should identify the key genes involved in HA-induced cold resistance in melon seedlings.

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