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Hydrogen Sulfide Interacts with 5-Aminolevulinic Acid to Enhance the Antioxidant Capacity of Pepper (*Capsicum annuum* L.) Seedlings under Chilling Stress

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Abstract: 5-Aminolevulinic acid (ALA) is the precursor of tetrapyrrole synthesis, and hydrogen sulfide (H_2S) is a gas signal molecule. Studies have shown that exogenous ALA and H_2S can alleviate abiotic stress. This study evaluated the roles of ALA and H_2S and their interactions in regulating antioxidant activity in pepper seedlings under chilling stress. Chilling stress significantly inhibited the growth of pepper seedlings and increased the amounts of hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\bullet-}$), and malondialdehyde (MDA). ALA and/or H_2S increased the activities of superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT). Moreover, ALA and/or H_2S enhanced the ascorbate (AsA)-glutathione (GSH) cycle by increasing the contents of AsA and GSH, the ratio of AsA to dehydroascorbic acid and GSH to glutathione disulfide increased, and the activities of ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR) and monodehydroascorbate reductase (MDHAR) also increased. At the transcriptional level, ALA and/or H_2S upregulated the expressions of *CaSOD*, *CaPOD*, *CaCAT*, *CaAPX*, *CaGR*, *CaDHAR*, and *CaMDHAR* in seedlings under chilling stress. ALA and/or H_2S also reduced the contents of H_2O_2 , $O_2^{\bullet-}$ and MDA, eventually mitigating the inhibitory effects of chilling stress on pepper seedling growth. The combination of ALA and H_2S had a better effect than ALA or H_2S alone. Moreover, ALA and H_2S interact to regulate the oxidative stress response of pepper seedlings under chilling stress.

Keywords: antioxidase activities; ascorbate-glutathione cycle; low temperature; reactive oxygen species; transcriptional regulation

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1. Introduction

Pepper (*Capsicum annuum* L.) is an important vegetable and seasoning crop, which is typically thermophilous plant [1,2]. The normal growth temperature of pepper is 20–30 °C, and the growth and development will be hindered if the temperature is below 15 °C [2]. In winter and spring, chilling is the main abiotic stress that limits the growth, development and yield of pepper [1]. Chilling stress seriously affects plant growth, development, and metabolic pathways, leading to poor germination, leaf yellowing, reduced leaf expansion, wilt, and delayed seedling development [3]. Chilling stress can also change the structure and function of cell membranes [4]. Meanwhile, plants produce a large amount of reactive oxygen species (ROS), including singlet oxygen (1O_2), hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and superoxide anion ($O_2^{\bullet-}$) under chilling stress, which cause cell oxidative damage when produced in excess [5–7]. ROS have a bidirectional effect in vivo. On the one hand, ROS led to irreversible DNA damage and cell death; on the other, ROS regulate plant growth and stress as signaling molecules [8,9]. ROS also regulate stomatal behavior, pathogen defense, and programmed cell death [8,10,11]. Plants have a complex and complete antioxidant system to remove ROS and protect cells from oxidative damage [6,10]. The antioxidant systems in living organisms mainly depend on antioxidant molecules, antioxidant enzymes, and some osmotic regulatory substances [11,12].

Antioxidant molecules include glutathione (GSH), ascorbate (AsA), and α -tocopherol [11]. Among the antioxidant pathways, the AsA-GSH cycle is considered to be the most important, which is regulated by four key enzymes: glutathione reductase (GR), ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) [13]. Antioxidant enzymes include catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), APX, and other antioxidant enzymes [11,13].

ALA, a precursor of the synthesis of tetrapyrrole (chlorophyll, heme and cyrheme), is a novel plant growth regulator and plays important roles in plants [14–16]. ALA enhances plant growth, yield, and abiotic stress tolerance by regulating antioxidant mechanisms, photosynthesis, and nutrient uptake [17]. Exogenous ALA can also ameliorate oxidative stress caused by low-temperature stress in tomato, cucumber, soybean, mung bean and maize [5,15,18–20]. Moreover, under chilling stress, the activities of antioxidant enzymes (APX, GPX, SOD, and POD) increased in rice [21]. Excepting the activities of antioxidant enzymes (SOD, CAT, APX, DHAR, and GR), the ratio of GSH to glutathione disulfide (GSSG) and AsA to dehydroascorbic acid (DHA) in tomato were also significantly increased under chilling [13].

As a vital gas signaling molecule, hydrogen sulfide (H_2S), plays a crucial role in promoting tolerance to environmental stress and mitigating the adverse effects of stress on plants [22,23]. H_2S is involved in the regulation of plant growth, development, and resistance to abiotic stress (cold, salinity, drought, and heavy metals) through interactions with ROS, hormones, and other molecular signals [7,24]. The effect of H_2S on abiotic stress is generally related to oxidative stress, and previous studies have shown that H_2S and ROS have both antagonistic and synergistic effects when regulating plant response to stress [25,26]. Chilling stress induces a short and rapid increase in endogenous H_2S in plants [7,24,27]. The application of sodium hydrosulfide (NaHS; an H_2S precursor) increased the activities of antioxidant enzymes in cucumber under chilling stress [27].

ALA has been reported to interact with nitric oxide (NO) to initiate cold tolerance in plants, and acts upstream of NO to respond to chilling tolerance by activating antioxidant defenses and plasma membrane (PM) H^+ -ATPase and maintaining Na and K homeostasis in the roots of *Elymus nutans* [28]. ALA also interacts with downstream signals (H_2O_2 , NO, and jasmonic acid (JA)) to induce chilling tolerance in tomato [29]. H_2S was also shown to induce cold tolerance in cucumber through interactions with indole-3-acetic acid (IAA) and salicylic acid (SA) [27,30], and enhance the salt tolerance of alfalfa seed germination through the NO pathway [31]. H_2S may also be a downstream signaling molecule in heat tolerance induced by NO in maize seedlings [32]. The chromium tolerance of foxtail millet was found to be enhanced by the interaction of H_2S with calcium [33].

ALA or H_2S can regulate abiotic stress in plants by enhancing antioxidant activity. However, few studies focused on the relationship between H_2S and ALA in abiotic stress. In this study, we evaluated the roles of ALA and/or H_2S and their interactions in regulating oxidative stress in pepper seedlings by measuring the ROS content and malondialdehyde (MDA), antioxidant enzyme activity and transcription level of genes encoding their synthesis, and the AsA-GSH cycle under chilling stress.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

Pepper seeds (Hangjiao 2, Shenzhou Iypeng, Tianshui, China) were soaked in distilled water at 55 °C for 15 min and then soaked at room temperature for 6 h. After this, the seeds were spread evenly on a wet towel and incubated in the dark for 3–5 days at 28 °C. After germinating, seeds were planted in the substrate (vermiculite: peat: cow dung = 1:1:1) for growth. The pepper seedlings were grown in a light incubator under the following conditions: relative humidity = 50–75%; temperature = 28 °C/18 °C (12 h light/12 h dark); and light intensity = 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

2.2. Treatments and Experimental Design

Pepper seedlings were treated with chilling stress when they had grown six true leaves. Exogenous substances were sprayed before chilling stress: 1 mmol/L NaHS (a producer of H_2S) and 150 $\mu\text{mol/L}$ hypotaurine (HT; an H_2S scavenger) were sprayed on the leaves 24 h before chilling stress, and 25 mg/L ALA solution was sprayed on the leaves 12 h after the spraying of HT and NaHS, following our previous research [34]. Each treatment contained 50 seedlings and each pot was sprayed with 10 mL of exogenous substances. The experimental treatments were as follows: regular training (RT; 28 °C/18 °C day/night temperature and 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity); chilling stress (CK, 10 °C/5 °C day/night temperature and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity); ALA (25 mg/L ALA and chilling stress); ALA + HT (25 mg/L ALA + 150 $\mu\text{mol/L}$ HT and chilling stress); ALA + NaHS (25 mg/L ALA + 1 mmol/L NaHS and chilling stress); and NaHS (1 mmol/L NaHS and chilling stress). All chilling stress was performed under the same conditions (10 °C/5 °C day/night temperature and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity); the photoperiod of all treatments was 12 h/12 h (light/dark), and the chilling stress was applied for a total of seven days.

2.3. Biomass, Morphology, and Physiological Indexes

After one week of chilling stress, 6 plants were selected from each treatment to measure the plant height, stem thickness, blade quantity, shoot fresh weight, and root fresh weight. The functional leaves of five well-grown seedlings functional leaves were mixed as one sample, and the physiological indexes (the contents of H_2O_2 , $\text{O}_2^{\bullet-}$, malondialdehyde (MDA), GSH, GSSG, AsA, DHA, and antioxidant enzyme activities) were determined with three biological replicates of each using assay kits (Comin Biotechnology, Suzhou, China) after 24 h of chilling stress. Absorbance was determined using a UV-1780 spectrophotometer (Shimadzu, Kyoto, Japan).

2.4. Histochemical Staining and the Contents of H_2O_2 , $\text{O}_2^{\bullet-}$, and MDA

The 3,3-diaminobenzidine (DAB) and nitroblue tetrazolium (NBT) staining were used to detect the accumulation of hydrogen peroxide and superoxide anion, respectively, after 24 h of low-temperature stress, and the detection method referred to in Reference [1].

The contents of H_2O_2 , $\text{O}_2^{\bullet-}$, and MDA were measured using assay kits (Comin Biotechnology, Suzhou, China) following the manufacturer's instructions. H_2O_2 and titanium sulfate combine to form titanium peroxide, which exhibits absorption at 415 nm. MDA and thiobarbituric acid are condensed into a red product with a maximum absorption at 532 nm. The samples (0.1 g) were ground in ice bath with 1 mL of MDA extract, and then centrifuged at 8000× g and 4 °C for 10 min. The supernatant was used to determine MDA content following the manufacturer's instruction. The absorbance at 600 nm was measured, and the MDA content was calculated by the difference in the absorbance. $\text{O}_2^{\bullet-}$ reacts with hydroxylamine hydrochloride to form NO^{2-} , which reacts with p-aminobenzene sulfonic acid and naphthalamine to form the azo compound of Mangrove Bay, which has a characteristic absorption peak at 530 nm. The content of $\text{O}_2^{\bullet-}$ was calculated according to the difference between the measured value and the blank value.

2.5. Antioxidant Enzyme Activities

Fresh leaf samples (0.1 g) were ground in an ice bath with 1 mL of extract solution (0.1 mol/L sodium phosphate buffer, pH = 7.8). The homogenate was then centrifuged at 8000× g and 4 °C for 10 min. The supernatant was used to determine the activities of SOD, POD, and CAT using assay kits (Comin Biotechnology, Suzhou, China) following the manufacturer's instructions. SOD can remove $\text{O}_2^{\bullet-}$, which can react with WST-8 to form formazan, which shows absorption at 450 nm. The activity of POD (EC 1.11.1.7) was determined according to manufacturer's instructions by determining absorbance values at 470 nm for 1 and 2 min. at 470 nm. The CAT (EC 1.11.1.6) activity was determined by initial and 2-min absorbance values at 240 nm according to the manufacturer's instructions. To determine the APX and GR activities after chilling stress for 24 h, 0.1 g leaf sample was

ground with pre-cooled extract and then centrifuged at $13,000\times g$ and $4\text{ }^{\circ}\text{C}$ for 20 min. The supernatant was used to determine the activities of APX and GR following the manufacturer's instructions. Similarly, the activities of DHAR and MDHAR were determined using a common extract, which was centrifuged at $8000\times g$ and $4\text{ }^{\circ}\text{C}$ for 10 min. Next, the activities of DHAR and MDHAR were determined using the supernatant according to the manufacturer's instructions.

2.6. The Contents of GSH, GSSG, AsA, and DHA

The contents of GSH, GSSG, AsA, and DHA were measured using assay kits (Comin Biotechnology, Suzhou, China). Fresh leaf samples (0.1 g) were homogenized with 1 mL of GSH extract solution in an ice bath and centrifuged at $8000\times g$ for 10 min. Supernatant (100 μL) was used to determine the GSH and GSSG contents following the manufacturer's instructions. Similarly, AsA and DHA can also be extracted together. Fresh leaf samples (0.1 g) were homogenized with 1 mL AsA extract solution (2 mol/L acetic acid buffer, $\text{pH} = 4.5$) in an ice bath and centrifuged at $12,000\times g$ for 20 min. The supernatant was then used to determine the contents of AsA and DHA following the manufacturer's instructions. Finally, the GSH/GSSG and AsA/DHA ratios were calculated.

2.7. RNA Extraction and qRT-PCR

Total RNA from pepper leaves was isolated using an RNA extraction kit (Tiangen, Beijing, China). The quality and concentration of RNA were determined by ultramicro spectrophotometry (Pultron, Charlott, USA) and agarose gel electrophoresis. Genomic DNA was removed and RNA was reverse-transcribed using kit (TransGen Biotech, Beijing, China), and the quality and concentration of cDNA were determined by ultramicro spectrophotometry (Pultron, Charlott, USA). Then, cDNA was diluted to 150 ng/ μL and used for qRT-PCR using kits (TransGen Biotech, Beijing, China), following the manufacturer's instructions. qRT-PCR was performed using a QuantStudio5 RT-PCR System (Thermo Fisher Scientific Inc., Waltham, USA). Each reaction included three biological and three technical replicates. *Actin* of pepper (Gene ID: 107875540) was used as the internal reference gene. All primers, including *Actin* and cDNA, were pretested for efficiency and reaction efficiency. The gene sequences were searched from the NCBI website (<https://www.ncbi.nlm.nih.gov/gene>, 1 February 2022). Primers were designed for qRT-PCR, and the sequences are shown in Table 1.

Table 1. Primers used for the qRT-PCR.

Genes	Accessions	Forward Primer	TM	Reverse Primer	TM
<i>CaSOD</i>	AF036936.2	GCTTCATCACCAGAAACATCATCAGAC	57.79	ATGACCTCCGCCATTGAACCTTGATAG	58.83
<i>CaPOD</i>	FJ596178.1	GCCATTACTGCTAGGGACTCTGTTG	59.71	GAAGTAGGAGGAGGAATGCTGCTATTG	58.98
<i>CaCAT</i>	AB007190.1	TTAACGCTCCCAAGTGTGCTCATC	59.68	GGGCAAATAATCCACCTCTCATCG	60.08
<i>CaAPX</i>	DQ002888.1	GAGCAGTTTCCCACTCTCTATG	59.47	CATCAGGTCTCCAGTAACTTCAACAG	59.01
<i>CaGR</i>	AY547351.1	GTTAATTCAACTGGATGGACCAAGATG	58.11	ATTCCTGGACGATGAGCCCTACTAC	60.19
<i>CaDHAR2</i>	JW079767.1	CCGTCAGTAAATCCTTGCTCTTCAG	59.17	TACCCAAATCCCTCTCTCGTTACTCC	59.69
<i>CaMDHAR</i>	AY652702.1	GAGCAAGACCACTACGACTCTATTC	59.07	AACATCACCTACAGCGTACACATCAG	58.60
<i>CaActin</i>	XM_016722297.1	GTCTTCCATCGTCCACAGG	58.38	GAAGGGCAAAGGTTCAACA	55.84

2.8. Statistical Analysis

The data in this study are shown as the mean $\pm$ standard error of three independent experiments. A statistical analysis was conducted using SPSS 22.0 (SPSS Institute Inc., Chicago, IL, USA). Duncan's test was used to assess significant differences among the means at a probability level of 5%. All figures were created in Origin version 9 (OriginLab Institute Inc., Northampton, MA, USA).

3. Results

3.1. Effects of ALA and/or H_2S on the Growth of Pepper Seedlings under Chilling Stress

Plant phenotypes were observed and photographed under chilling stress for 24 h. After 24 h of chilling, low-temperature alone (CK) and ALA + HT treatments showed severe

wilting, while ALA and NaHS alone showed lighter wilting than CK. ALA combined with NaHS (ALA + NaHS) had almost no wilting (Figure 1A).

Figure 1. Effects of ALA and/or H₂S on the phenotype under chilling stress and the growth under chilling stress in pepper seedlings: (A) the phenotype under chilling stress for 24 h, (B) plant height for 1 week, (C) stems thickness for 1 week, (D) blade quantity for 1 week, (E) shoot fresh weight for 1 week, and (F) root fresh weight for 1 week. NaHS: a producer of H₂S; HT: an H₂S scavenger. RT: 28 °C/18 °C day/night temperature and 300 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity; CK: 10 °C/5 °C day/night temperature and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity; ALA: 25 mg/L ALA +10 °C/5 °C day/night temperature and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity; ALA + HT: 25 mg/L ALA + 150 $\mu\text{mol/L}$ HT + 10 °C/5 °C day/night temperature and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity; ALA + NaHS: 25 mg/L ALA + 1 mmol/L NaHS +10 °C/5 °C day/night temperature and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity; NaHS: 1 mmol/L NaHS +10 °C/5 °C day/night temperature and 100 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ light intensity. The error bars are the standard errors for 6 independent tests. Different letters show significant differences ($p < 0.05$) based on Duncan's test.

The plant height, stem thickness, blade quantity, shoot fresh weight, and root fresh weight of seedlings were measured after one week of chilling stress. These indexes significantly decreased during chilling stress for one week (Figure 1B–F). Compared to chilling stress alone (CK), the application of exogenous ALA increased the plant height, stem thickness, blade quantity, shoot fresh weight, and root fresh weight by 9.2%, 18.7%, 27.8%, 56.8%, and 36.9%, respectively. Simultaneously, compared to CK, the application of exogenous NaHS increased the plant height, stem thickness, and shoot fresh weight by 8.06%, 11.2%, and 40.4%, respectively (Figure 1B,C,E). However, there was no significant difference in the blade quantity and root fresh weight between NaHS and CK treatments. In addition, compared to CK, ALA + NaHS treatment increased the plant height, stem thickness, shoot fresh weight, and root fresh weight by 15.4%, 20.5%, 59.9% and 35.0%,

respectively (Figure 1B,C,E,F). Meanwhile, the plant height, stem thickness, blade quantity, shoot fresh weight, and root fresh weight were significantly reduced in the group treated with ALA + HT compared to the ALA (Figure 1B–F). These results indicate that the application of exogenous ALA and/or H₂S significantly alleviated the growth-inhibiting effects of chilling stress in pepper seedlings. Meanwhile, HT inhibited the ability of ALA to alleviate the effects of chilling stress on pepper growth.

3.2. Effects of ALA and/or H₂S on H₂O₂, O₂^{•−}, and MDA under Chilling Stress

DAB and NBT staining reflected the accumulation of hydrogen peroxide and superoxide anion. CK and ALA + HT treatments showed deeper staining compared with other treatments, while ALA, NaHS and ALA + NaHS treatments showed a significantly lighter color than CK (Figure 2A,B). In addition, the H₂O₂ and O₂^{•−} contents and were measured after 24 h of chilling stress. Under chilling stress (CK), the contents of H₂O₂ and O₂^{•−} increased by 138.6% and 34.2% compared to RT, respectively (Figure 2D,E). For the ALA, NaHS, and ALA + NaHS treatments, the H₂O₂ contents were decreased by 17.7%, 40.9%, and 25.6%, compared to CK, respectively, while the contents of O₂^{•−} were decreased by 16.7%, 14.5%, and 19.5%, respectively (Figure 2D,E). The MDA content indirectly reflects the degree of membrane lipid peroxidation. Compared to CK, the ALA, NaHS, and ALA + NaHS treatments decreased the content of MDA by 24.0%, 28.0%, and 25.6%, respectively (Figure 2C). No significant differences were observed in the contents of H₂O₂, O₂^{•−}, and MDA between ALA + HT and CK under chilling stress (Figure 2C–E).

3.3. Effects of ALA and/or H₂S on the Activities of SOD, POD, and CAT under Chilling Stress

To evaluate the antioxidant activity of pepper seedlings under chilling stress, we measured the activities of SOD, POD, and CAT along with the relative expression levels of the related genes. Compared to CK, exogenous ALA increased the activities of SOD, POD, and CAT by 127.4%, 54.4%, and 58.1% and the relative expression levels of *CaSOD*, *CaPOD*, and *CaCAT* by 10.1-, 30.7-, and 7-fold, respectively (Figure 3A–F). Compared to CK, exogenous H₂S significantly increased the activities of SOD, POD, and CAT by 124.4%, 37.6%, and 39.7%, the relative expression levels of *CaSOD*, *CaPOD*, and *CaCAT* by 17.8-, 23-, and 4.9-fold, respectively (Figure 3A–F). ALA + NaHS significantly increased the activities of SOD, POD, and CAT by 115.3%, 48.9%, and 50.77%, and the relative expression levels of *CaSOD*, *CaPOD*, and *CaCAT* by 16.3-, 29.5-, and 6.6-fold compared to CK, respectively (Figure 3A–F). Compared to the ALA group, the activities of SOD, POD, and CAT in the ALA + HT group were decreased by 34.9%, 29.4%, and 27.7%, respectively (Figure 3A–C). Moreover, the relative expression levels of *CaSOD*, *CaPOD*, and *CaCAT* in the ALA + HT group were significantly lower than those in the ALA group (Figure 3D–F).

3.4. Effects of ALA and/or H₂S on the Contents of GSH and GSSG under Chilling Stress

The contents of GSH and GSSG after 24 h of chilling stress are shown in Figure 4. Chilling stress significantly reduced the content of GSH compared to RT, and ALA alleviated this effect of chilling stress (Figure 4A). However, NaHS treatment had no significant effect on GSH content compared to CK (Figure 4A). In contrast, chilling stress significantly increased the content of GSSG. Compared to CK, the ALA, ALA + HT, ALA + NaHS, and NaHS treatments decreased the GSSG content by 37.7%, 19.4%, 48.7%, and 51.5%, respectively (Figure 4B). Moreover, chilling stress significantly reduced the GSH/GSSG ratio compared to RT (Figure 4C). The ALA, ALA + NaHS, and NaHS treatments increased the GSH/GSSG ratio by 1.3-, 1.3-, and 1.1-fold compared to CK, respectively (Figure 4C). No significant difference in GSH/GSSG ratio was observed between the ALA + HT and CK groups (Figure 4C). These results suggest that ALA increased the GSH/GSSG ratio in pepper seedlings subjected to chilling stress for 24 h by increasing the GSH content and decreasing the GSSG content; meanwhile, H₂S increased the GSH/GSSG ratio primarily by decreasing the GSSG content.

Figure 2. Effects of ALA and/or H₂S on DAB staining (A), NBT staining (B), the contents of MDA (C), H₂O₂ (D), and O₂^{•-} (E) under chilling stress for 24 h. The error bars are the standard errors for three independent tests. Different letters show significant differences ($p < 0.05$) based on Duncan's test.

3.5. Effects of ALA and/or H₂S on the Contents of AsA and DHA under Chilling Stress

As shown in Figure 5, chilling stress markedly reduced the content of AsA and increased the content of DHA (Figure 5A,B). Compared to CK, the ALA, NaHS, ALA + NaHS, and ALA + HT treatments increased the content of AsA by 17.9%, 20.3%, 24.4%, and 5.6%, respectively (Figure 5A). Meanwhile, compared to CK, the ALA, NaHS, and ALA + NaHS treatments decreased the content of DHA by 33.7%, 34.8%, and 44.7%, respectively (Figure 5B), and the AsA/DHA ratio increased by 77.4%, 84.3%, and 124.4%, respectively (Figure 5C). No significant differences in DHA content and AsA/DHA ratio were found between the ALA + HT and CK groups (Figure 5B,C).

Figure 3. Effects of ALA and/or H₂S on SOD (A), POD (B), and CAT (C) activities and the relative expressions of *CaSOD* (D), *CaPOD* (E), and *CaCAT* (F) under chilling stress for 24 h. The error bars are the standard errors for three independent tests. Different letters show significant differences ($p < 0.05$) based on Duncan's test.

Figure 4. Effects of ALA and/or H₂S on the contents of GSH (A), GSSG (B), and GSH/GSSG ratio (C) under chilling stress for 24 h. The error bars are the standard errors for three independent tests. Different letters show significant differences ($p < 0.05$) based on Duncan's test.

Figure 5. Effects of ALA and/or H₂S on the contents of AsA (A), DHA (B), and AsA/DHA ratio (C) under chilling stress for 24 h. The error bars are the standard errors for three independent tests. Different letters show significant differences ($p < 0.05$) based on Duncan's test.

3.6. Effects of ALA and/or H₂S on APX and GR Activity under Chilling Stress

Figure 6A shows the effects of ALA and/or H₂S on APX activity in pepper seedlings subjected to chilling stress for 24 h. APX activity was not significantly affected by 24 h of chilling stress (Figure 6A). However, compared to CK, the ALA, NaHS, and ALA + NaHS treatments increased the APX activity by 55.6%, 71.4% and 54.5%, respectively (Figure 6A). Meanwhile, the ALA, NaHS, and ALA + NaHS treatments increased the GR activity in pepper seedlings by 1.3-, 1.1-, and 1.2-fold, respectively (Figure 6B). In addition, the ALA, NaHS, and ALA + NaHS treatments increased the relative expression of *CaAPX* by 98.8%, 379.1%, and 159.8%, respectively (Figure 6C), and increased the relative expression of *CaGR* by 41.1%, 76.6%, and 57.1%, respectively (Figure 6D). No significant differences in the activities of APX and GR or in the relative expressions of *CaAPX* and *CaGR* were observed between the ALA + HT and CK groups (Figure 6A–D). These results suggest that exogenous ALA and/or H₂S enhanced the activities of APX and GR in pepper seedlings under chilling stress.

3.7. Effects of ALA and/or H₂S on DHAR and MDHAR Activity under Chilling Stress

To further study the effects of ALA and/or H₂S on the AsA-GSH cycle, we measured the activities of DHAR and MDHAR and the transcript levels of *CaDHAR* and *CaMDHAR*. Compared to CK, the application of ALA increased the activities DHAR and MDHAR by 29.8% and 53.9%, respectively, and enhanced the relative expressions of *CaDHAR* and *CaMDHAR* by 32.4% and 27.3%, respectively (Figure 7A–D). Meanwhile, compared to CK, the application of NaHS increased the activities of DHAR and MDHAR by 29.4% and 53.6%, respectively, and enhanced the relative expressions of *CaDHAR* by 31.9% and 22.4%, respectively (Figure 7A–C). Compared to CK, exogenous ALA + NaHS increased the activities of DHAR and MDHAR by 53.0% and 111.9%, respectively, and increased the relative expressions of *CaDHAR* and *CaMDHAR* by 265.7% and 187.0%, respectively (Figure 7A–D). There were no significant differences in the DHAR and MDHAR activities, or the relative expression levels of *CaDHAR* and *CaMDHAR* between the ALA + HT and CK groups (Figure 7A–D). These results suggest that ALA and H₂S enhanced the relative expressions of *CaAPX*, *CaGR*, *CaDHAR*, and *CaMDHAR* in pepper seedlings under chilling stress, with the combination of ALA and NaHS having the most significant effect.

Figure 6. Effects of ALA and/or H₂S on the activities of APX (A) and GR (B) and the relative expressions of *CaAPX* (C) and *CaGR* (D) under chilling stress for 24 h. The error bars are the standard errors for three independent tests. Different letters show significant differences ($p < 0.05$) based on Duncan's test.

Figure 7. Effects of ALA and/or H₂S on the activities of DHAR (A) and MDHAR (B) and the relative expressions of *CaDHAR* (C) and *CaMDHAR* (D) under chilling stress for 24 h. The error bars are the standard errors for three independent tests. Different letters show significant differences ($p < 0.05$) based on Duncan's test.

4. Discussion

Low temperature is an important limiting factor in the production of pepper [1,35]. In our study, chilling stress significantly reduced the stem diameter, plant height, the number of leaves, and fresh weight of pepper seedlings. The addition of exogenous ALA significantly mediated the decreases in plant height, hypocotyl diameter, root length, plant dry weight, leaf area, and strong seedling index of cucumber and maize seedlings caused by low-temperature stress [5,20]. H₂S has also been found to increase the growth parameters and biomass of cucumber [36], and rice seedlings [37]. Similar to these findings, in this study, treatment with ALA and/or H₂S increased the stem diameter, plant height, leaves number, and fresh weight in pepper seedlings under chilling stress. In addition, the effects of the ALA and ALA + NaHS treatments were more significant than those of the NaHS treatment. However, the application of HT (an H₂S scavenger) significantly reduced the promotion of ALA to the growth of pepper seedlings under chilling stress, suggesting that H₂S was involved in ALA to alleviate the inhibition on the growth of pepper seedlings caused by low temperature.

The metabolic pathways in plant organelles become disordered in adverse environments, leading to the production and accumulation of ROS. The presence of excessive ROS induces oxidative stress, which results in the oxidation of cell components, affects the integrity of organelles, and impedes metabolic activity [38]. ROS play important roles in improving tolerance to abiotic stresses and maintaining normal plant growth [8]. Low temperature results in an imbalance between the light and dark reactions of photocollaboration, causing the photosynthetic electron transport chain to release more superoxide (O₂^{•−}) via electron transfer to oxygen as a substitute electron acceptor [39]. MDA is one of the final decomposition products of membrane lipid peroxidation, and an increase in MDA content is generally considered to be indicative of membrane lipid peroxidation, which often occurs in plant tissues under abiotic stress [40,41]. In this study, cold storage increased the contents of H₂O₂, O₂^{•−}, and MDA in pepper seedlings. This is consistent with the results of Tang et al. (2021), who found that serious lipid peroxidation of the cell membrane occurred under chilling stress [1,42]. SOD is a scavenger of O₂^{•−} and the main enzyme producing H₂O₂. The overexpression of Mn-SOD was found to enhance the salt tolerance of *Arabidopsis thaliana* [43]. Moreover, exogenous SOD at chilling stress can improve the cold tolerance of plants [44]. CAT is the main scavenger of H₂O₂, and POD eliminates H₂O₂, phenols, and amines [45]. Zhao et al. (2020) reported that chilling stress increased the contents of H₂O₂ and MDA, along with the activities of SOD, POD, GR, and APX, in mung bean, while exogenous ALA enhanced the activities of antioxidant enzymes [19]. The transcript levels of SOD, APX, and MDHAR were found to decrease slightly under chilling stress in *A. thaliana* [46]. Consistent with these previous studies, our results indicate that low temperature increased the contents of H₂O₂, O₂^{•−}, and MDA in pepper seedlings. However, unlike the results reported in mung beans, low temperature did not increase the activities of SOD, POD, and CAT in our study. This discrepancy may be related to the different responses of different plants to chilling stress [39]. Under chilling stress, the application of ALA was found to increase the activities of SOD and CAT in pepper, maize, and cucumber, while it decreased the content of MDA [5]. Exogenous ALA also significantly reduced the contents of MDA and H₂O₂ and enhanced the activities of SOD, POD, CAT, APX, and GR in oilseed *rape* under cadmium (Cd) stress [47]. H₂S inhibited oxidative damage and membrane peroxidation by activating an antioxidant enzyme system after Cd treatment [48]. The synergistic effect of SA and H₂S enhanced the antioxidant system in maize [49]. Exogenous H₂S accumulation in hawthorn fruit also enhanced the activities of L-cysteine desulphydrase (LCD), D-cysteine desulphydrase (DCD), SOD, CAT, and APX along with the accumulation of AsA, resulting in reduced H₂O₂ accumulation after harvest under freezing damage [50]. These studies demonstrate that H₂S can participate in antioxidant stress response through over-sulfated antioxidant enzymes [25]. In the present study, ALA and/or H₂S significantly increased the activities of SOD, POD, CAT, APX, and GR and decreased the contents of H₂O₂, O₂^{•−}, and MDA in pepper seedlings

under chilling stress. In addition, H₂S and ALA significantly enhanced the transcription levels of antioxidant enzymes.

The AsA-GSH cycle is the primary ROS scavenging system in cells [51]. GSH is a small redox-active molecule that mainly exists in two stable forms, GSH and GSSG [52]. DHA is the reversible oxidized form of AsA. As a reducing agent of DHA, GSH participates in the AsA-GSH cycle. DHA can also maintain α -tocopherol and zeaxanthin in the reduced states and protect the proteins from denaturing via the oxidation of mercaptan groups, thereby protecting the integrity of the cell plasma membrane [53]. In normally growing plants, the GSH/GSSG ratio is high; exposure to adverse environmental conditions can alter this ratio, particularly by inducing an increase in GSSG [52]. In this study, ALA increased the content of GSH and decreased the content of GSSG in seedlings under chilling stress, causing the GSH/GSSG ratio to be similar to that under regular training. H₂S also increased the GSH/GSSG ratio by decreasing the content of GSSG. A high GSH/GSSG and/or AsA/DHA ratio may be the key to effectively ameliorating the accumulation of ROS induced by abiotic stress [27,54]. In our study, ALA and/or H₂S significantly increased the content of AsA and the AsA/DHA ratio, while they decreased the content of DHA. Treatment with ALA maintained the high AsA/DHA and GSH/GSSG ratios in *Brassica napus* under drought stress [55]. Similarly, H₂S was found to enhance the contents of AsA and GSH along with the enzyme activities in the AsA-GSH cycle in pea under arsenate stress [56].

APX catalyzes the reduction of H₂O₂ to H₂O by using AsA as an electron donor, resulting in the formation of DHA. DHAR reduces DHA to AsA via the electrons provided by GSH. NADPH reduces GSSG to GSH under the catalysis of GR. DHA is generated from MDHA. MDHAR catalyzes the reduction of MDHA to AsA. Thus, APX, GR, DHAR, and MDHAR are the key enzymes in the AsA-GSH cycle. The overexpression of chloroplast APX in tobacco was reported to improve tolerance to chilling stress [57]. The overexpression of GR in tobacco increased the level of GSH in leaves and enhanced the resistance to oxidative stress [58]. The overexpression of DHAR increased the fresh weight, seedling length, germination rate, and chlorophyll content in tomato under salt stress [59]. Sultana et al. (2012) found that the overexpression of MDHAR in a mangrove plant conferred salt tolerance to rice [60]. In addition, the overexpression of MDHAR gene in *A. thaliana* enhanced the stress tolerance of tobacco [61]. In cucumber, the application of ALA enhanced the activities of GR, APX, DHAR, and MDHAR, and upregulated the expressions of CAT and APX genes in roots and leaves, GR in roots under salt stress [62]. H₂S is involved in antioxidant activity at multiple levels, including transcriptional and post-translational modification [25]. Exogenous ALA significantly improved the gene expressions of antioxidant enzymes in *Brassica napus* under Cd stress [63]. The application of ALA stimulated the expressions of SOD, CAT, GR, and DHAR genes under lead stress in *Brassica juncea* [64]. In the present study, ALA and/or H₂S significantly increased the activities of APX, GR, DHAR, and MDHAR and enhanced the expression levels of *CaAPX*, *CaGR*, *CaDHAR*, and *CaMDHAR* in pepper seedlings under chilling stress, thereby enhancing the AsA-GSH pathway.

Our research shows that both ALA and H₂S enhanced the antioxidant capacity of pepper seedlings under chilling stress to improve their cold resistance. The ALA + HT treatment had significantly negative effects on the growth of pepper seedlings and antioxidant activity compared with ALA and ALA + NaHS treatments. The application of HT increased the accumulation of ROS and the content of MDA, and decreased the ascorbate (AsA)-glutathione (GSH) cycle and the activity of antioxidant enzymes by down-regulating the gene expression of antioxidant enzymes. These findings indicated that H₂S scavenging prevented ALA from regulating the antioxidant system and cold resistance of pepper seedlings, and ALA and H₂S interact to regulate the oxidative stress response of pepper seedlings under chilling stress.

5. Conclusions

The exogenous application of ALA and/or H₂S to pepper seedlings under chilling stress enhanced the antioxidant defense system. ALA and/or H₂S increased the activities of antioxidant enzymes by increasing the enzyme transcription levels. The transcription of GR, APX, DHAR, and MDHAR enhanced the AsA-GSH cycle, ultimately reducing the contents of O₂^{•−}, H₂O₂, and MDA and alleviating the negative effects of low-temperature stress. Meanwhile, a combination of ALA and H₂S had better effects than treatment with ALA or H₂S alone. HT reversed the effects of ALA, indicating that the antioxidant effect of ALA requires the participation of H₂S.

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References

1. Tang, C.; Xie, J.; Lv, J.; Li, J.; Zhang, J.; Wang, C.; Liang, G. Alleviating damage of photosystem and oxidative stress from chilling stress with exogenous zeaxanthin in pepper (*Capsicum annuum* L.) seedlings. *Plant Physiol. Biochem.* **2021**, *162*, 395–409. [\[CrossRef\]](#) [\[PubMed\]](#)
2. Guo, W.L.; Chen, R.G.; Gong, Z.H.; Yin, Y.X.; Li, D.W.; Pandey, G.K. Suppression Subtractive Hybridization Analysis of Genes Regulated by Application of Exogenous Abscissic Acid in Pepper Plant (*Capsicum annuum* L.) Leaves under Chilling Stress. *PLoS ONE* **2013**, *8*, e66667. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Yadav, S.K. Cold stress tolerance mechanisms in plants. A review. *Agron. Sustain. Dev.* **2010**, *30*, 515–527. [\[CrossRef\]](#)
4. Lu, Y.; Hu, Y.; Snyder, R.L.; Kent, E.R. Tea leaf's microstructure and ultrastructure response to low temperature in indicating critical damage temperature. *Inf. Processing Agric.* **2019**, *6*, 247–254. [\[CrossRef\]](#)
5. Anwar, A.; Yan, Y.; Liu, Y.; Li, Y.; Yu, X. 5-Aminolevulinic Acid Improves Nutrient Uptake and Endogenous Hormone Accumulation, Enhancing Low-Temperature Stress Tolerance in Cucumbers. *Int. J. Mol. Sci.* **2018**, *19*, 3379. [\[CrossRef\]](#)
6. Ma, X.; Chen, C.; Yang, M.; Dong, X.; Lv, W.; Meng, Q. Cold-regulated protein (SICOR413IM1) confers chilling stress tolerance in tomato plants. *Plant Physiol. Biochem.* **2018**, *124*, 29–39. [\[CrossRef\]](#)
7. Singh, S.; Kumar, V.; Kapoor, D.; Kumar, S.; Singh, S.; Dhanjal, D.S.; Datta, S.; Samuel, J.; Dey, P.; Wang, S.; et al. Revealing on hydrogen sulfide and nitric oxide signals co-ordination for plant growth under stress conditions. *Physiol. Plant.* **2020**, *168*, 301–317. [\[CrossRef\]](#)
8. Huang, H.; Ullah, F.; Zhou, D.X.; Yi, M.; Zhao, Y. Mechanisms of ROS Regulation of Plant Development and Stress Responses. *Front. Plant Sci.* **2019**, *10*, 800. [\[CrossRef\]](#)
9. Miller, G.; Suzuki, N.; Ciftci-Yilmaz, S.; Mittler, R. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant Cell Environ.* **2010**, *33*, 453–467. [\[CrossRef\]](#)
10. Gill, S.S.; Tuteja, N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol. Biochem.* **2010**, *48*, 909–930. [\[CrossRef\]](#)
11. Schippers, J.; Nguyen, H.M.; Lu, D.; Schmidt, R.; Mueller-Roeber, B. ROS homeostasis during development: An evolutionary conserved strategy. *Cell. Mol. Life Sci.* **2012**, *69*, 3245. [\[CrossRef\]](#) [\[PubMed\]](#)
12. Seth, C.S.; Remans, T.; Keunen, E.; Jozefczak, M.; Gielen, H.; Opdenakker, K.; Weyens, N.; Vangronsveld, J.; Cuypers, A. Phytoextraction of toxic metals: A central role for glutathione. *Plant Cell Environ.* **2012**, *35*, 334–346. [\[CrossRef\]](#) [\[PubMed\]](#)
13. Kaya, C.; Ashraf, M.; Akram, N.A. Hydrogen sulfide regulates the levels of key metabolites and antioxidant defense system to counteract oxidative stress in pepper (*Capsicum annuum* L.) plants exposed to high zinc regime. *Environ. Sci. Pollut. Res.* **2018**, *25*, 12612–12618. [\[CrossRef\]](#) [\[PubMed\]](#)
14. Zulfiqar, F.; Hancock, J.T. Hydrogen sulfide in horticulture: Emerging roles in the era of climate change. *Plant Physiol. Biochem.* **2020**, *155*, 667–675. [\[CrossRef\]](#) [\[PubMed\]](#)

15. Liu, T.; Hu, X.; Zhang, J.; Zhang, J.; Du, Q.; Li, J. H₂O₂ mediates ALA-induced glutathione and ascorbate accumulation in the perception and resistance to oxidative stress in *Solanum lycopersicum* at low temperatures. *BMC Plant Biol.* **2018**, *18*, 34. [\[CrossRef\]](#)
16. Wu, Y.; Liao, W.; Dawuda, M.M.; Hu, L.; Yu, J. 5-Aminolevulinic acid (ALA) biosynthetic and metabolic pathways and its role in higher plants: A review. *Plant Growth Regul.* **2018**, *87*, 357–374. [\[CrossRef\]](#)
17. Balestrasse, K.B.; Tomaro, M.L.; Batlle, A.; Noriega, G.O. The role of 5-aminolevulinic acid in the response to cold stress in soybean plants. *Phytochemistry* **2010**, *71*, 2038–2045. [\[CrossRef\]](#)
18. Akram, N.A.; Ashraf, M. Regulation in Plant Stress Tolerance by a Potential Plant Growth Regulator, 5-Aminolevulinic Acid. *J. Plant Growth Regul.* **2013**, *32*, 663–679. [\[CrossRef\]](#)
19. Rhaman, M.S.; Imran, S.; Karim, M.M.; Chakroborty, J.; Mahamud, M.A.; Sarker, P.; Tahjib-Ul-Arif, M.; Robin, A.H.K.; Ye, W.; Murata, Y.; et al. 5-aminolevulinic acid-mediated plant adaptive responses to abiotic stress. *Plant Cell Rep.* **2021**, *40*, 1451–1469. [\[CrossRef\]](#)
20. Liu, T.; Xu, J.; Zhang, J.; Li, J.; Hu, X. Exogenous 5-aminolevulinic acid pretreatment ameliorates oxidative stress triggered by low-temperature stress of *Solanum lycopersicum*. *Acta Physiol. Plant.* **2018**, *40*, 1–11. [\[CrossRef\]](#)
21. Zhao, J.J.; Zhou, N.; Feng, N.J.; Zheng, D.F. Effects of 5-aminolevulinic acid on osmotic adjustment and antioxidant system in mung bean under chilling stress. *Biol. Plant.* **2020**, *64*, 736–743. [\[CrossRef\]](#)
22. Wang, Y.; Li, J.; Gu, W.; Qian, Z.; Tian, L.; Guo, S.; Wei, S. Exogenous application of 5-aminolevulinic acid improves low-temperature stress tolerance of maize seedlings. *Crop Pasture Sci.* **2018**, *69*, 587–593. [\[CrossRef\]](#)
23. Sheteiwy, M.; Shen, H.; Xu, J.; Guan, Y.; Song, W.; Hu, J. Seed polyamines metabolism induced by seed priming with spermidine and 5-aminolevulinic acid for chilling tolerance improvement in rice (*Oryza sativa* L.) seedlings. *Environ. Exp. Bot.* **2017**, *137*, 58–72. [\[CrossRef\]](#)
24. Wang, R. Gasotransmitters: Growing pains and joys. *Trends Biochem. Sci.* **2014**, *39*, 227–232. [\[CrossRef\]](#) [\[PubMed\]](#)
25. Gotor, C.; García, I.; Aroca, N.; Laureano-Marín, A.; Romero, L.C. Signaling by hydrogen sulfide and cyanide through posttranslational modification. *J. Exp. Bot.* **2019**, *70*, 4251–4265. [\[CrossRef\]](#)
26. Du, X.; Jin, Z.; Liu, D.; Yang, G.; Pei, Y. Hydrogen sulfide alleviates the cold stress through MPK4 in *Arabidopsis thaliana*. *Plant Physiol. Biochem.* **2017**, *120*, 112–119. [\[CrossRef\]](#)
27. Chen, T.; Tian, M.; Han, Y. Hydrogen sulfide: A multi-tasking signal molecule in the regulation of oxidative stress responses. *J. Exp. Bot.* **2020**, *71*, 2862–2869. [\[CrossRef\]](#)
28. Li, Z.; Zhu, Y.; He, X.; Yong, B.; Peng, Y.; Zhang, X.; Xiao, M.; Yan, Y.; Huang, L.; Nie, G. The hydrogen sulfide, a downstream signaling molecule of hydrogen peroxide and nitric oxide, involves spermidine-regulated transcription factors and antioxidant defense in white clover in response to dehydration. *Environ. Exp. Bot.* **2018**, *161*, 255–264. [\[CrossRef\]](#)
29. Fu, P.; Wang, W.; Hou, L.; Liu, X. Hydrogen sulfide is involved in the chilling stress response in *Vitis vinifera* L. *Acta Soc. Bot. Pol.* **2013**, *82*, 295–302. [\[CrossRef\]](#)
30. Zhang, X.W.; Liu, F.J.; Zhai, J.; Li, F.D.; Bi, H.G.; Ai, X.Z. Auxin acts as a downstream signaling molecule involved in hydrogen sulfide-induced chilling tolerance in cucumber. *Planta* **2020**, *251*, 69. [\[CrossRef\]](#)
31. Fu, J.J.; Chu, X.T.; Sun, Y.F.; Xu, Y.F.; Hu, T.M. Involvement of nitric oxide in 5-aminolevulinic acid-induced antioxidant defense in roots of *Elymus nutans* exposed to cold stress. *Biol. Plant.* **2016**, *60*, 1–10. [\[CrossRef\]](#)
32. Liu, T.; Xu, J.; Li, J.; Hu, X. NO is involved in JA- and H₂O₂-mediated ALA-induced oxidative stress tolerance at low temperatures in tomato. *Environ. Exp. Bot.* **2019**, *161*, 334–343. [\[CrossRef\]](#)
33. Pan, D.Y.; Fu, X.; Zhang, X.W.; Liu, F.J.; Bi, H.G.; Ai, X.Z. Hydrogen sulfide is required for salicylic acid-induced chilling tolerance of cucumber seedlings. *Protoplasma* **2020**, *257*, 1543–1557. [\[CrossRef\]](#) [\[PubMed\]](#)
34. Wang, Y.; Li, L.; Cui, W.; Xu, S.; Shen, W.; Wang, R. Hydrogen sulfide enhances alfalfa (*Medicago sativa*) tolerance against salinity during seed germination by nitric oxide pathway. *Plant Soil* **2012**, *351*, 107–119. [\[CrossRef\]](#)
35. Li, Z.G.; Yang, S.Z.; Long, W.B.; Yang, G.X.; Shen, Z.Z. Hydrogen sulfide may be a novel downstream signal molecule in nitric oxide-induced heat tolerance of maize (*Zea mays* L.) seedlings. *Plant Cell Environ. Plant Cell Environ.* **2013**, *36*, 1564–1572. [\[CrossRef\]](#)
36. Fang, H.; Jing, T.; Liu, Z.; Zhang, L.; Jin, Z.; Pei, Y. Hydrogen sulfide interacts with calcium signaling to enhance the chromium tolerance in *Setaria italica*. *Cell Calcium Int. Interdiscip. Forum Res. Calcium* **2014**, *56*, 472–481. [\[CrossRef\]](#)
37. Wang, H.; Liu, Z.; Luo, S.; Li, J.; Zhang, J.; Li, L.; Xie, J. 5-Aminolevulinic acid and hydrogen sulphide alleviate chilling stress in pepper (*Capsicum annuum* L.) seedlings by enhancing chlorophyll synthesis pathway. *Plant Physiol. Biochem.* **2021**, *167*, 567–576. [\[CrossRef\]](#)
38. Korkmaz, A.; Korkmaz, Y.; Demirkiran, A.R. Enhancing chilling stress tolerance of pepper seedlings by exogenous application of 5-aminolevulinic acid. *Environ. Exp. Bot.* **2010**, *67*, 495–501. [\[CrossRef\]](#)
39. Wu, G.X.; Cai, B.B.; Zhou, C.F.; Li, D.D.; Bi, H.G.; Ai, X.Z. Hydrogen sulfide-induced chilling tolerance of cucumber and involvement of nitric oxide. *J. Plant Biol. Res.* **2016**, *5*, 58–69.
40. Duan, B.; Ma, Y.; Jiang, M.; Yang, F.; Ni, L.; Lu, W. Improvement of photosynthesis in rice (*Oryza sativa* L.) as a result of an increase in stomatal aperture and density by exogenous hydrogen sulfide treatment. *Plant Growth Regul.* **2014**, *75*, 33–44. [\[CrossRef\]](#)
41. Suzuki, N.; Koussevitzky, S.; Mittler, R.; Miller, G. ROS and redox signalling in the response of plants to abiotic stress. *Plant Cell Environ.* **2012**, *35*, 259–270. [\[CrossRef\]](#) [\[PubMed\]](#)

42. Dreyer, A.; Dietz, K.-J. Reactive Oxygen Species and the Redox-Regulatory Network in Cold Stress Acclimation. *Antioxidants* **2018**, *7*, 169. [[CrossRef](#)] [[PubMed](#)]
43. Qiu, Z.B.; Li, J.T.; Zhang, M.M.; Bi, Z.Z.; Li, Z.L. He–Ne laser pretreatment protects wheat seedlings against cadmium-induced oxidative stress. *Ecotoxicol. Environ. Saf.* **2013**, *88*, 135–141. [[CrossRef](#)] [[PubMed](#)]
44. Chen, Q.; Zhang, M.; Shen, S. Effect of salt on malondialdehyde and antioxidant enzymes in seedling roots of Jerusalem artichoke (*Helianthus tuberosus* L.). *Acta Physiol. Plant.* **2010**, *33*, 273–278. [[CrossRef](#)]
45. Wu, Y.; Hu, L.; Liao, W.; Mujitaba Dawuda, M.; Lyu, J.; Xie, J.; Feng, Z.; Calderón-Urrea, A.; Yu, J. Foliar application of 5-aminolevulinic acid (ALA) alleviates NaCl stress in cucumber (*Cucumis sativus* L.) seedlings through the enhancement of ascorbate-glutathione cycle. *Sci. Hortic.* **2019**, *257*, 108761. [[CrossRef](#)]
46. Wang, Y.; Yin, Y.; Jia, C.; Wang, X. Transgenic Arabidopsis overexpressing Mn-SOD enhanced salt-tolerance. *Plant Sci.* **2004**, *167*, 671–677. [[CrossRef](#)]
47. Wang, X. Effects of exogenous SOD on physiological characteristics of *Petunia hybrida* under low temperature stress. *Seed* **2021**, *40*, 108–112.
48. Juszczak, I.; Cvetkovic, J.; Zuther, E.; Hinch, D.K.; Baier, M. Natural Variation of Cold Deacclimation Correlates with Variation of Cold-Acclimation of the Plastid Antioxidant System in Arabidopsis thaliana Accessions. *Front. Plant Sci.* **2016**, *7*, 305. [[CrossRef](#)]
49. Ali, B.; Huang, C.R.; Qi, Z.Y.; Ali, S.; Daud, M.K.; Geng, X.X.; Liu, H.B.; Zhou, W.J. 5-Aminolevulinic acid ameliorates cadmium-induced morphological, biochemical, and ultrastructural changes in seedlings of oilseed rape. *Environ. Sci. Pollut. Res.* **2013**, *20*, 7256–7267. [[CrossRef](#)]
50. Luo, S.; Tang, Z.; Yu, J.; Liao, W.; Xie, J.; Lv, J.; Feng, Z.; Dawuda, M.M. Hydrogen sulfide negatively regulates Cd-induced cell death in cucumber (*Cucumis sativus* L.) root tip cells. *BMC Plant Biol.* **2020**, *20*, 480. [[CrossRef](#)]
51. Li, Z.G. Synergistic effect of antioxidant system and osmolyte in hydrogen sulfide and salicylic acid crosstalk-induced heat tolerance in maize (*Zea mays* L.) seedlings. *Plant Signal. Behav.* **2015**, *10*, e1051278. [[CrossRef](#)] [[PubMed](#)]
52. Aghdam, M.S.; Mahmoudi, R.; Razavi, F.; Rabiei, V.; Soleimani, A. Hydrogen sulfide treatment confers chilling tolerance in hawthorn fruit during cold storage by triggering endogenous H₂S accumulation, enhancing antioxidant enzymes activity and promoting phenols accumulation. *Sci. Hortic.* **2018**, *238*, 264–271. [[CrossRef](#)]
53. Avashthi, H.; Pathak, R.K.; Pandey, N.; Arora, S.; Mishra, A.K.; Gupta, V.K.; Ramteke, P.W.; Kumar, A. Transcriptome-wide identification of genes involved in Ascorbate–Glutathione cycle (Halliwell–Asada pathway) and related pathway for elucidating its role in antioxidative potential in finger millet (*Eleusine coracana* (L.)). *3 Biotech* **2018**, *8*, 499. [[CrossRef](#)]
54. Rahantaniaina, M.S.; Tuzet, A.; Mhamdi, A.; Noctor, G. Missing links in understanding redox signaling via thiol/disulfide modulation: How is glutathione oxidized in plants? *Front. Plant Sci.* **2013**, *4*, 477. [[CrossRef](#)] [[PubMed](#)]
55. Fotopoulos, V.; Ziogas, V.; Tanou, G.; Molassiotis, A. Involvement of AsA/DHA and GSH/GSSG Ratios in Gene and Protein Expression and in the Activation of Defence Mechanisms Under Abiotic Stress Conditions. In *Ascorbate–Glutathione Pathway and Stress Tolerance in Plants*; Springer: Dordrecht, The Netherlands, 2010; pp. 265–302.
56. Szalai, G.; Kells, T.; Galiba, G.; Kocsy, G. Glutathione as an Antioxidant and Regulatory Molecule in Plants under Abiotic Stress Conditions. *J. Plant Growth Regul.* **2009**, *28*, 66–80. [[CrossRef](#)]
57. Liu, D.; Pei, Z.F.; Naeem, M.S.; Ming, D.F.; Liu, H.B.; Khan, F.; Zhou, W.J. 5-Aminolevulinic Acid Activates Antioxidative Defence System and Seedling Growth in *Brassica napus* L. under Water Deficit Stress. *J. Agron. Crop Sci.* **2011**, *197*, 284–295. [[CrossRef](#)]
58. Singh, V.P.; Singh, S.; Kumar, J.; Prasad, S.M. Hydrogen sulfide alleviates toxic effects of arsenate in pea seedlings through up-regulation of the ascorbate-glutathione cycle: Possible involvement of nitric oxide. *J. Plant Physiol.* **2015**, *181*, 20–29. [[CrossRef](#)]
59. Yabuta, Y.; Motoki, T.; Yoshimura, K.; Takeda, T.; Ishikawa, T.; Shigeoka, S. Thylakoid membrane-bound ascorbate peroxidase is a limiting factor of antioxidative systems under photo-oxidative stress. *Plant J.* **2002**, *32*, 915–925. [[CrossRef](#)]
60. Broadbent, P.; Creissen, G.P.; Kular, B.; Wellburn, A.R.; Mullineaux, P.M. Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. *Plant J.* **1995**, *8*, 247–255. [[CrossRef](#)]
61. Li, Q.; Li, Y.; Li, C.; Yu, X. Enhanced Ascorbic Acid Accumulation through Overexpression of Dehydroascorbate Reductase Confers Tolerance to Methyl Viologen and Salt Stresses in Tomato. *Czech J. Genet. Plant Breed.* **2012**, *48*, 74–86. [[CrossRef](#)]
62. Sultana, S.; Khew, C.Y.; Morshed, M.M.; Namasivayam, P.; Napis, S.; Ho, C.L. Overexpression of monodehydroascorbate reductase from a mangrove plant (AeMDHAR) confers salt tolerance on rice. *J. Plant Physiol.* **2012**, *169*, 311–318. [[CrossRef](#)] [[PubMed](#)]
63. Eltayeb, A.E.; Kawano, N.; Badawi, G.H.; Kaminaka, H.; Sanekata, T.; Shibahara, T.; Inanaga, S.; Tanaka, K. Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. *Planta* **2007**, *225*, 1255–1264. [[CrossRef](#)] [[PubMed](#)]
64. Zhen, A.; Bie, Z.L.; Huang, Y.; Liu, Z.X.; Fan, M.L. Effects of 5-aminolevulinic acid on the H₂O₂-content and antioxidative enzyme gene expression in NaCl-treated cucumber seedlings. *Biol. Plant.* **2012**, *56*, 566–570. [[CrossRef](#)]