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Hydrogen Sulfide Increases Drought Tolerance by Modulating Carbon and Nitrogen Metabolism in Foxtail Millet Seedlings

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Abstract: Hydrogen sulfide (H_2S), a novel gas signaling molecule, has been shown to enhance plant resistance to various abiotic stresses. Here, we investigated the effect of sodium hydrosulfide (NaHS, a H_2S donor) on the growth, photosynthetic parameters, and enzyme activities related to carbon and nitrogen metabolism, as well as the levels of carbohydrates and nitrogen metabolites in foxtail millet seedlings subjected to drought stress conditions in pots. The findings revealed that drought stress led to a significant 41.2% decline in the total dry weight (DW) after 12 days of treatment, whereas plants treated with NaHS showed a lesser reduction of 18.7% in total DW. Under drought stress, exogenous NaHS was found to enhance carbon metabolism in foxtail millet seedlings by significantly enhancing photosynthetic capacity, starch, and sucrose content. Additionally, exogenous NaHS was observed to improve nitrogen metabolism by substantially increasing soluble protein content, nitrogen assimilate activity, and synthesis of nitrogen-containing compounds in foxtail millet seedlings. In summary, the exogenous application of NaHS stimulated seedling growth and enhanced drought resistance in foxtail millet by modulating carbon and nitrogen metabolism processes affected by drought stress.

Keywords: hydrogen sulfide; drought stress; foxtail millet; carbon and nitrogen metabolism

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1. Introduction

Given the backdrop of escalating global water scarcity, drought has exerted a profound impact on world agricultural production in recent years [1]. Drought events in agricultural production are marked by their extensive geographic distribution, frequent incidence, and extended duration, leading to adverse impacts on crops at various stages of their growth cycle. Drought hinders plant growth and development, disturbs physiological and metabolic processes, and may cause DNA damage, ultimately resulting in significant decreases in crop yields or, in extreme cases, death [2–4]. Hence, enhancing crop resilience to drought is crucial for maintaining stable yields and successful harvests.

Hydrogen sulfide (H_2S) serves as a crucial signaling molecule in various physiological and pathological processes within plant systems. The release of H_2S gas by plants such as soybeans and cucumbers were first observed in 1978, establishing H_2S as the third most significant gas signal after NO and CO [5]. Due to its small size, H_2S can easily pass-through biological membranes and modulate plant cellular homeostasis without the need for specific receptors [6]. A substantial body of research has demonstrated that H_2S exerts regulatory influence over a multitude of vital physiological processes in plants. Specifically, the exogenous H_2S has been found to enhance photosynthesis activity in plants with high chlorophyll content, as evidenced by research conducted by Parveen et al. [7] and Liu et al. [8]. Additionally, the use

of H_2S donors has been found to effectively mitigate decay and mildew in postharvest fruits, leading to an extension of their storage duration, as reported by Ali et al. [9]. Furthermore, H_2S has been shown to confer resistance to plants against various abiotic stresses, including drought, cold, high salinity, heavy metal toxicity, and UV radiation, thereby offering significant protection against damage [10,11]. H_2S has been identified as a novel signaling molecule crucial for enhancing plant resistance to drought. It has the ability to stimulate the expression of *DES1*, further leading to the sulfhydrylation of *OST1/SNF1*-associated protein kinase (*SnRK2.6*), subsequently accelerating stomatal closure [12]. Furthermore, H_2S plays a role in responding to drought stress by modulating ion channels that safeguard cell membranes, either directly or indirectly. [13]. Nonetheless, a more comprehensive investigation is necessary to elucidate the precise mechanisms through which H_2S contributes to enhanced drought tolerance in plants.

The process of carbon and nitrogen metabolism among plants is a highly coordinated and unified process, which is crucial for regulating plant morphogenesis and life activities [14]. Plants require a sufficient nitrogen supply to facilitate the assimilation of CO_2 via photosynthesis for the production of carbohydrate nutrients. Additionally, a substantial quantity of fixed carbon is essential for nitrogen metabolism to serve as a carbon skeleton acceptor, along with a significant amount of ATP and NADPH to support the requisite biochemical processes. Achieving a harmonious balance between the assimilation capacities of carbon and nitrogen is imperative for enhancing crop resilience to stress, as highlighted by Ren et al. [15]. Carbon and nitrogen metabolites regulate plant response to environmental signals by regulating gene expression, enzyme and transporter activity related to carbon and nitrogen transport [16]. Therefore, maintaining equilibrium in carbon and nitrogen metabolism during drought stress is essential for promoting plant development and increasing crop productivity.

Foxtail millet (*Setaria italica* L.) ranks among the small-scale coarse grain crops with the largest cultivated area and highest total yield [17]. This crop is utilized for both its grain and grass, with grain grass and grain bran essential components for high-quality feed. Furthermore, it features a modest genome size of approximately 420 Mb, a brief life cycle, and rapid reproduction, rendering it a valuable model crop for investigating stress resistance [18]. Drought stress is recognized as the primary factor restricting foxtail millet yield and growth in agricultural production [19,20]. Hence, it is crucial to explore practical strategies to enhance the drought resistance of foxtail millet. Previous studies on H_2S in improving plant tolerance to drought primarily focuses on enhancing antioxidant capacity, regulating stomatal closure, and increasing photosynthetic efficiency [15,21]. However, the influence of H_2S on regulating metabolic processes in drought-stressed seedlings has received less attention. Photosynthesis is intricately linked to the metabolic processes of carbon and nitrogen, with interdependent regulatory mechanisms in plants. Here, we hypothesized that H_2S could enhance foxtail millet drought tolerance by alleviating the inhibitory effects of drought stress on carbon and nitrogen metabolism. This study examined the effects of exogenous NaHS on carbon and nitrogen metabolism in foxtail millet under water stress, shedding light on the interplay between H_2S, drought tolerance, and carbon and nitrogen metabolism.

2. Materials and Methods

2.1. Plant Materials and Drought and NaHS Treatment

The Jingu 21, a drought-sensitive variety, was provided from the Industrial Crop Institute of the Shanxi Academy of Agricultural Sciences in Taiyuan, China. Seeds that were uniform in size, color, and fullness were carefully selected. The seeds were disinfected using a 1% sodium hypochlorite solution for a duration of 10 min and then rinsed three times with sterile water. Subsequently, the treated seeds were planted in plastic pots measuring 17 cm in diameter and 22 cm in height, filled with 3 kg of loess. The application rates of N, P_2O_5, and K_2O were 0.24, 0.18, and 0.24 g kg^{-1} dried soil, respectively. To prevent evaporation, perlite was used to cover the surface of the soil. The artificial climate chamber was set up as follows: the temperature was 28 °C daytime/23 °C night, the photoperiod was light for 14 h and darkness for 10 h, the light intensity was 1000 μmol m^{-2} s^{-1}, and the relative humidity was 45–55%.

Foxtail millet seedlings were treated with 100 μM NaHS or distilled water at the four-leaf stage, followed by exposure to natural drought stress conditions 12 h later using a weighing and water control method [22]. Four treatment groups were established: (1) normal water supply with foliar spray of distilled water, (2) normal water supply with 100 μM NaHS, (3) drought stress with a foliar spray of distilled water, and (4) drought stress with 100 μM NaHS. Each treatment included 30 pots that were randomly placed. Sampling was conducted during the hours of 9:00–11:00 on the 6th and 12th days during the drought, specifically the fourth fully developed leaf and root system.

2.2. Determination of Biomass and Leaf Area

Following the induction of drought stress in foxtail millet seedlings during the middle (6 days) and late (12 days) stages, a total of six foxtail millet plants exhibiting uniform growth were selected for each treatment. Leaf length and width were measured with a ruler to calculate the total leaf area of the plant. Subsequently, the samples underwent a 30-min drying period at 105 °C, followed by further drying at 80 °C until a constant weight was achieved. The dry weight of the entire plant was determined using an analytical balance (TF2003, Heng Ji, Shang Hai, China). Subsequently, the shoots and roots were separated to determine their individual dry weights. Each treatment was replicated six times for statistical analysis.

2.3. Extraction and Determination of H_2S

The concentration of H_2S was quantified utilizing the methylene blue method as analyzed by Xiao et al. [23]. A total of 0.1 g of fresh leaf and root samples were taken and centrifugated with 0.9 mL 20 mmol·L^{-1} Tris-HCl buffer (pH = 8.0) in the ice bath for 20 min at 4 °C 2000× *g*. Subsequently, 0.5 mL of supernatant combined with 2 mL of extract, to which a specific quantity of zinc acetate was added. This mixture was then supplemented with 0.1 mL of 30 mM $FeCl_3$ solution and 0.1 mL of 20 mM N, N-dimethyl-p-phenylenediamine. After 15 min, a 670 nm wavelength was used to measure the absorbance. Each treatment was replicated three times for statistical analysis.

2.4. Determination of Gas Exchange Parameters, Chlorophyll Content, Fv/Fm, and Relative Water Content

The net photosynthetic rate (Pn), stomatal conductance (Gs), and transpiration rate (Tr) of the fourth fully expanded leaf were assessed using the LI-6800 infrared gas analyzer (LI-COR, Lincoln, NE, USA). The conditions during the measurements included synthetically effective radiation of 1000 μmol $m^{-2}s^{-1}$, humidity at 50%, leaf temperature of 25 °C, airflow at 500 μmol s^{-1}, and CO_2 concentration at 400 ppm. The pulsed amplitude modulated chlorophyll fluorescence system (Imaging PAM; Walz, Effeltrich, Germany) was utilized to assess the chlorophyll fluorescence parameters (Fv/Fm) of labeled leaves. The concentration of chlorophyll-a/b was determined using the formula proposed by Lichtenthaler [24]. The total chlorophyll concentration, as well as the chlorophyll-a/chlorophyll-b ratio were subsequently calculated. The fifth leaf, fully expanded and devoid of veins, was sectioned into small segments, weighed by fresh weight (FW) using a precision balance, involved with distilled water for 6 h in order to determine its saturated weight (TW), and subsequently subjected to drying at 70 °C for 24 h to ascertain its dry weight (DW). Leaf relative water content (RWC; %) was determined using the following equation. Six biological replicates were measured for each treatment.

$$\mathrm{RWC} = \frac{\mathrm{FW} - \mathrm{DW}}{\mathrm{TW} - \mathrm{DW}} \times 100$$

2.5. Enzyme Extraction and Activity Assay

The enzymatic activity of ribulose bisphosphate carboxylase (Rubisco) was assessed at 340 nm using the method described by Liu et al. [25]. The activity of ADP-glucose pyrophosphorylase (AGPase) was determined as outlined by Schaffer et al. [26]. AGPase

activity was quantified in milligrams per hour of increased NADPH per gram of fresh sample. Frozen leaf samples weighing 0.2 g were homogenized in 2 mL of 100 mM Tris-HCl buffer (pH = 7.0), followed by centrifugation at 10,000× *g* for 10 min at 4 °C. The activities of sucrose phosphate synthase (SPS), sucrose synthase (SuSy) and invertase (INV) in the supernatant were determined according to the method of Hu et al. [27]. SPS and SuSy were expressed as milligrams of sucrose produced per gram of fresh sample per hour. INV represents enzyme activity in milligrams of glucose and fructose (reducing sugar) produced per gram of fresh sample per hour. The activity of nitrate reductase (NR) was determined according to the method described by Barro et al. [28], with NR units defined as the amount of nitrogen dioxide produced per gram of fresh sample per hour. Glutamine synthetase (GS) activity was determined by O 'Neal and Joy [29], with GS enzyme activity reported in milligrams of gamma-glutamyl isohydroxyl acid produced per gram of fresh sample per hour. The activity of glutamate synthase (GOGAT) was assessed following the protocol outlined by Matoh and Takahashi [30], while the activity of glutamate dehydrogenase (GDH) activity was determined according to Loyola-Vargas and de Jimenez [31]. GOGAT and GDH units were defined as the amount of NADH consumed per gram of fresh sample per hour. Each treatment was replicated three times for statistical analysis.

2.6. Quantitative Analysis of Carbohydrates

The contents of sucrose, fructose, and glucose were quantified in a 0.2 g dry leaf sample following the methodology outlined Xu et al. [32]. Starch contents were determined using the procedures described by Hansen and Moller [33]. Specifically, 0.1 g of leaf dry samples were combined with 1 mL of an 80% ethanol solution, subjected to a water bath at 80 °C for 30 min, and centrifuged at 4000× *g* for 10 min at room temperature. This process was repeated twice. Subsequently, 0.4 mL of distilled water was added to the resulting precipitation and heated at 100 °C for 15 min. Following a 15-min stirring period, 0.4 mL of a 9.2 M $HClO_4$ solution was introduced to the precipitation. The reaction solution was centrifuged at room temperature at 4000× *g* 10 min and the supernatant were obtained. A 0.4 mL 4.6 M $HClO_4$ solution was added to the precipitation, stirred for 15 min, added 1 mL distilled water, centrifuged for 10 min, and combined with the supernatant. The precipitate was washed with distilled water twice, and the supernatant was filled with distilled water to 1 mL. A 0.1 mL extract solution was combined with 0.25 mL sulfate–anthrone reagent (ice water bath) and heated in a 100 °C water bath for 10 min. The absorbance at a wavelength of 620 nm was subsequently measured. Each treatment was replicated three times.

2.7. Determination of the Content of Metabolites Related to Nitrogen Metabolism

Leaf freeze-dried samples weighing 0.1 g were individually collected to determine the contents of free amino acids, soluble protein, NH_4^+ and NO_3^-. Free amino acids were quantified by grinding 0.1 g freeze-dried leaves into 1 mL 50 mM phosphate buffer (pH = 7.8) and centrifuging them in the ice bath for 20 min at 4 °C, 12,000× *g*. The content of free amino acids was determined following the protocol outlined Hajlaoui et al. [34]. The soluble protein content was quantified following the procedure outlined by Zhou et al. [35], while the ammonium nitrogen (NH_4^+) content was determined through indigo blue spectrophotometry described by Bräutigam et al. [36]. The content of nitrate nitrogen (NO_3^-) was assessed using the nitrosalicylic acid method detailed by Patterson et al. [37]. Each treatment was replicated three times.

2.8. Quantitative Real-Time PCR (qRT-PCR)

The foxtail millet leaves subjected to drought stress for a duration of 12 days were ground into a fine powder utilizing a mortar that had been pre-chilled with liquid nitrogen. Total RNA extraction was performed according to the RNAisoPlus kit of Baori Medical Biotechnology Co., Ltd (Beijing, China). Samples with a 260/280 ratio between 1.9 and 2.1 were selected and stored at −80 °C in a cryogenic refrigerator (DW-86L626, Haier, Beijing, China). cDNA was synthesized using Primescript™ RT gDNA Eraser (Perfect Real Time)

kit from Baori Medical Biotechnology. Subsequently, real-time primers were designed for CDS sequences using Primer Premier 5 software, as detailed in Supplemental Table S1 and compounded by Sangon Bioengineering Co., Ltd. (Shanghai, China). The specificity of the primers was validated by the analysis of the solubility curve obtained through scanning. The reaction utilized the TB Green® mixer Premix Ex Taq™ II (Tli RNaseH Plus) kit from Baori Medical Biotechnology Co., Ltd. (Beijing, China), and the Bio-rad CFX96 Touch real-time fluorescence quantitative instrument. The reaction system and procedure were displayed in Supplemental Tables S2 and S3. The internal reference SiActin (Seita.5G464000) was employed. The gene expression of each sample was determined by the $2^{-\Delta\Delta Ct}$ method.

2.9. Statistical Analysis

Statistical analysis was performed using SPSS 25.0 (IBM, Chicago, IL, USA) utilizing one-way ANOVA. Treatment differences were evaluated for significance using the least significant difference (LSD) test ($p < 0.05$). Graphical representations were generated using Sigmaplot 12.0.

3. Results

3.1. Effects of Exogenous NaHS on the Growth of Foxtail Millet under Drought Stress

NaHS treatment had no significant effect on the foxtail millet seedling growth under adequate irrigation conditions (Figure 1). Drought stress had a notable inhibitory effect on the growth of foxtail millet seedlings, resulting in a significant reduction in both shoot and root dry weights (Figure 1A,B). Following 6 and 12 days of drought treatment, the total dry weight of foxtail millet decreased by 22.5% and 41.2%, respectively, compared to the control treatment. Conversely, the total dry weight of foxtail millet under NaHS treatment increased by 27.5% and 38.4% compared to that under drought treatment (Figure 1C). Moreover, following 6 and 12 days of drought treatment, the leaf area of foxtail millet exhibited reductions of 20.1% and 31.8%, respectively, in comparison to the control group. Conversely, when subjected to drought conditions, the leaf area of foxtail millet treated with NaHS increased by 4.9% and 13.9%, respectively, as opposed to the drought treatment alone (Figure 1D). Exogenous NaHS could substantially increase the endogenous H_2S content in the foxtail millet roots and leaves (Figure 1E,F). Exogenous NaHS foliar spraying effectively alleviates drought stress on foxtail millet.

3.2. Effects of Exogenous NaHS on Carbon Assimilation of Foxtail Millet under Drought Stress

Under adequate irrigation conditions, NaHS had no significant effects on Pn, Gs, Tr, Fv/Fm, Rubisco activity, chlorophyll content and RWC of foxtail millet leaves. Compared with the control, the Pn, Gs, and Tr of foxtail millet leaves were significantly decreased under drought stress. In contrast, after 12 days of drought stress, plants treated with NaHS had 66.3%, 56.9%, and 42.2% higher Pn, Gs, and Tr, respectively, compared to drought-treated plants (Figure 2A,C,D). In addition, foxtail millet leaves treated with NaHS showed significantly higher Rubisco activity than drought plants. Following 6 and 12 days of drought stress exposure, the activity of Rubisco in foxtail millet leaves subjected to NaHS treatment exhibited a respective increase of 26.4% and 52.5% in comparison to drought-stressed plants (Figure 2B). The chlorophyll content of foxtail millet seedlings significantly decreased by 22.44% and 49.3% after 6 and 12 days of drought stress treatment, respectively, in contrast to the control group. However, the chlorophyll content of foxtail millet leaves treated with NaHS demonstrated a notable increase when compared to untreated drought-stressed plants, showing increments of 28.9% and 66.3% after 6 and 12 days of drought stress, respectively (Figure 3A). In response to water stress, the levels of chlorophyll a/b, RWC, and Fv/Fm in foxtail millet leaves exhibited a statistically significant decrease of 12%, 13%, and 15%, respectively, after a period of 12 days. NaHS treatment significantly increased chlorophyll a/b, RWC, and Fv/Fm of foxtail millet leaves (Figure 3B–D), indicating improved carbon assimilation under drought stress.

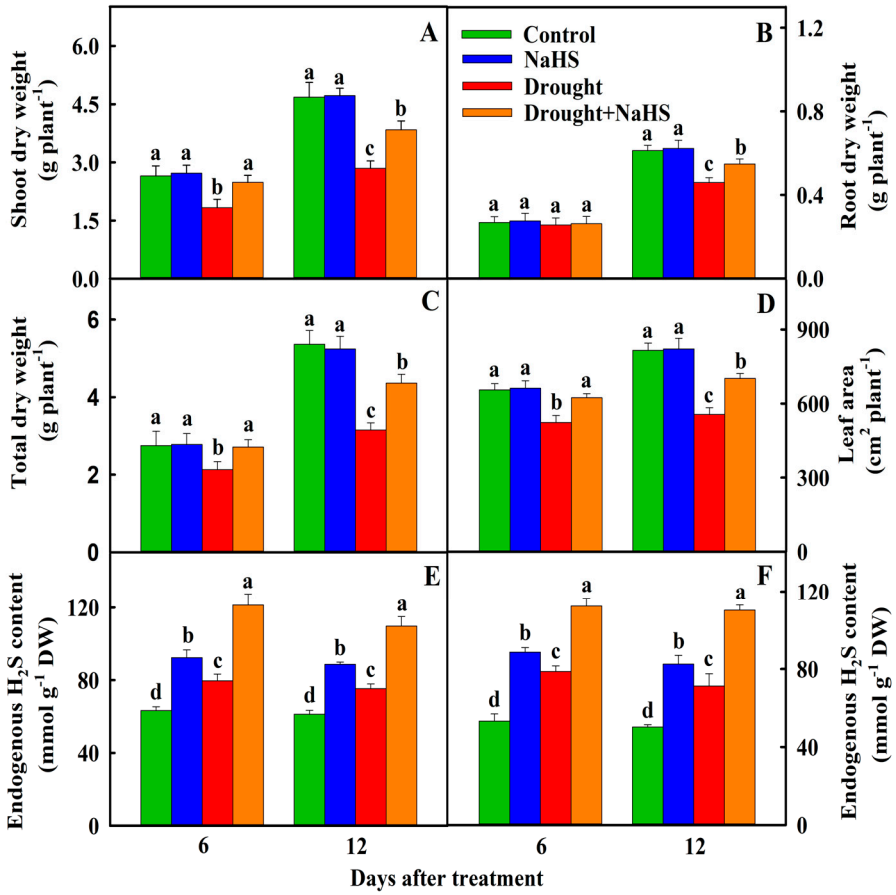

Figure 1. Effects of NaHS on the shoot dry weight (**A**), root dry weight (**B**), total plant dry weight (**C**), leaf area (**D**), leaf H_2S content (**E**), and root tip H_2S content (**F**) of foxtail millet at 6 and 12 days under drought stress. Data are mean ± s.e. (n = 3 for leaf H_2S content, root tip H_2S content; n = 6 for others). Values represented by distinct letters exhibit statistically significant differences ($p < 0.05$).

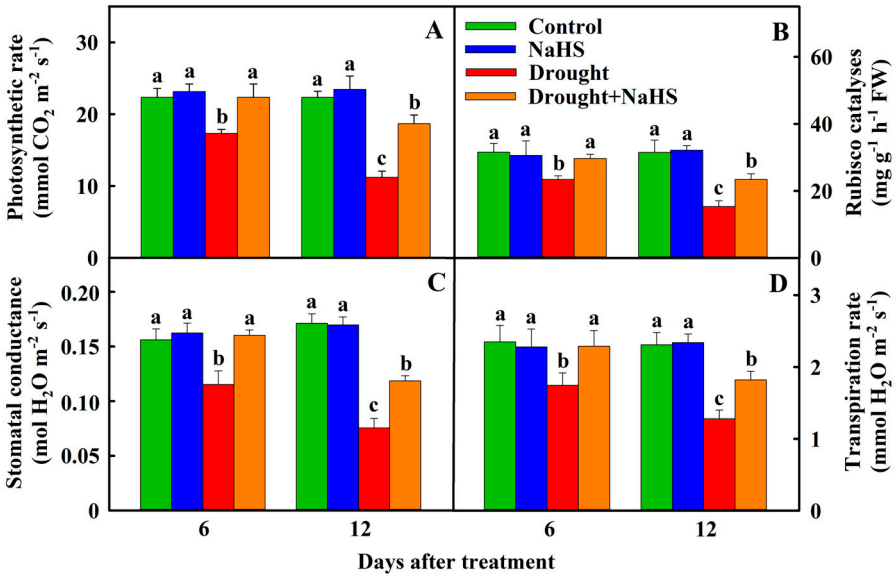

Figure 2. Effects of NaHS on the photosynthetic rate (**A**), Rubisco catalysis activity (**B**), stomatal conductance (**C**), and transpiration rate (**D**) of foxtail millet leaves at 6 and 12 days under drought stress. Data are mean ± s.e. (n = 6). Values represented by distinct letters exhibit statistically significant differences ($p < 0.05$).

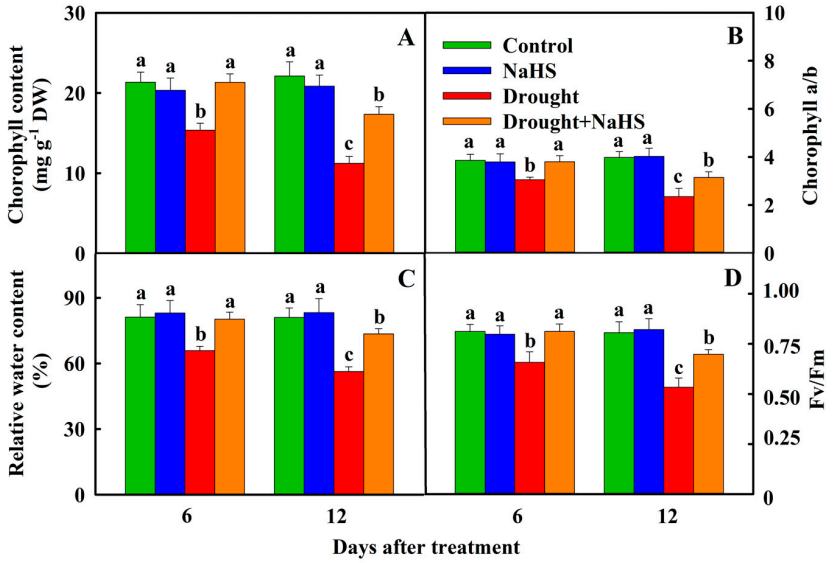

Figure 3. Effects of NaHS on the chlorophyll content (**A**), chlorophyll a/b (**B**), relative water content (**C**), and Fv/Fm (**D**) of foxtail millet leaves at 6 and 12 days under drought stress. Data are mean ± s.e. (n = 3). Values represented by distinct letters exhibit statistically significant differences ($p < 0.05$).

3.3. Effects of Exogenous NaHS on Carbon Metabolism of Foxtail Millet under Drought Stress

Under normal growth conditions, the addition of NaHS did not significantly affect AGPase, SPS, SuSy, INV, starch, sucrose, fructose, and glucose content in foxtail millet leaves. The effect of drought stress on starch synthesis in foxtail millet leaves is mainly due to the reduction of AGPase and SPS activities, which in turn reduces starch synthesis. The activities of AGPase and SPS in foxtail millet leaves decreased by 56.5% and 47.7% respectively compared to the control after 12 days of drought stress. In contrast, NaHS treatment only resulted in a 22.8% decrease in AGPase activity and a 24.9% decrease in SPS activity in foxtail millet leaves (Figure 4A,B). The activities of SuSy and INV, two important enzymes involved in sucrose hydrolysis in foxtail millet leaves, were higher under drought stress than in foxtail millet treated with NaHS (Figure 4C,D). In contrast, after 6 days and 12 days of drought stress, NaHS treatment increased the starch and sucrose contents in foxtail millet leaves (Figure 5A,B). In the absence of drought stress, foxtail millet leaves exhibited a significant increase in fructose and glucose content when not treated with NaHS. Following 6 and 12 days of drought stress, the fructose content in foxtail millet leaves treated with NaHS decreased by 22.7% and 28.6%, respectively, in comparison to drought-stressed plants. Similarly, the glucose content in foxtail millet leaves treated with NaHS decreased by 41.9% and 30.1% after 6 and 12 days of drought stress, respectively, compared to drought-stressed plants (Figure 5C,D). This shows that exogenous NaHS treatment can enhance the content of sucrose and starch under drought stress. The results indicated that treating foxtail millet with NaHS enhanced its ability to sequester carbon under water deficit conditions.

3.4. Effects of Exogenous NaHS on Nitrogen Assimilation and Nitrogen Metabolism of Foxtail Millet under Drought Stress

Under normal water supply conditions, the activity of the nitrogen assimilation enzyme (NR, GDH, GS, GOGAT) and the content of nitrogen metabolites (NH_4^+, NO_3^-, soluble protein, free amino acids) in foxtail millet leaves was not affected by NaHS. Following 6 and 12 days of drought stress treatment, the activity of NR in the leaves of foxtail millet seedlings exhibited a reduction of 27.4% and 46.7%, respectively, in comparison to the control group. Conversely, the NR activity in the leaves of foxtail millet seedlings subjected to NaHS treatment showed a significant increase when compared to the drought treatment, with increments of 34.3% and 46.8%, respectively (Figure 6A). Following 6 and 12 days of drought stress, the activity of GDH in foxtail millet leaves treated with NaHS exhibited a reduction of 30.9% and 23.2%, respectively, in

comparison to plants subjected solely to drought conditions (Figure 6B). Despite the application of NaHS, there were no noticeable differences in the activity levels of GS and GOGAT enzymes (Figure 6C,D), and NH_4^+ content between foxtail millet leaves treated with NaHS and those not under drought stress (Figure 7A). This suggests that different biological processes could produce the same level of NH_4^+ content. Foliar spraying NaHS significantly restored NO_3^- and soluble protein, levels in foxtail millet leaves under drought (Figure 7B,C). The free amino acids content in foxtail millet leaves increased by 28.4% and 89.5% after 6 and 12 days of drought stress, respectively. In contrast, the free amino acids content in foxtail millet leaves treated with NaHS experienced a decrease of 22.0% and 31.5%, respectively, when compared to drought-induced plants (Figure 7D). After being treated with NaHS, the plants exhibited higher NO_3^- content, GDH activity, and soluble protein content in comparison to non-treated plants.

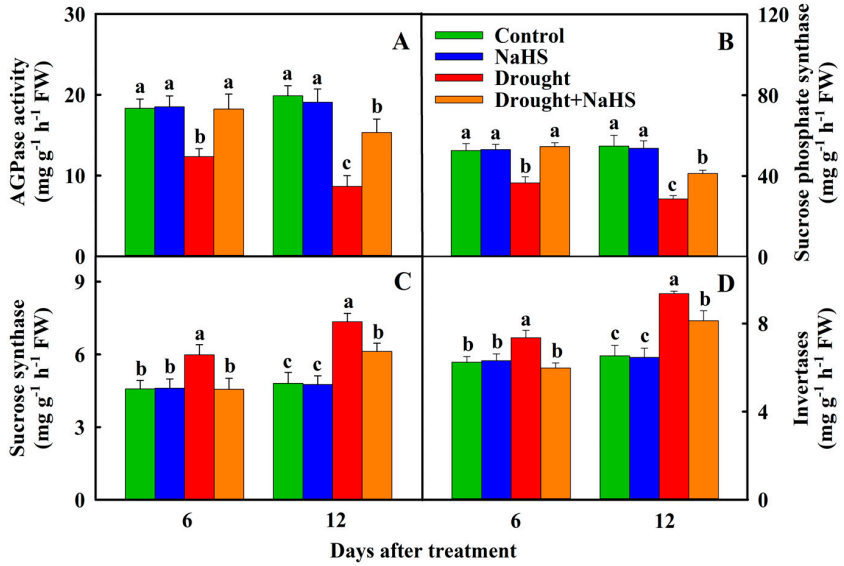

Figure 4. Effects of NaHS on the ADP-glucose pyrophosphorylase (AGPase) activity (**A**), sucrose phosphate synthase (SPS) activity (**B**), sucrose synthase (SS) activity (**C**), and invertases (INV) activity (**D**) in foxtail millet leaves at 6 and 12 days under drought stress. Data are mean ± s.e. (n = 3). Values represented by distinct letters exhibit statistically significant differences ($p < 0.05$).

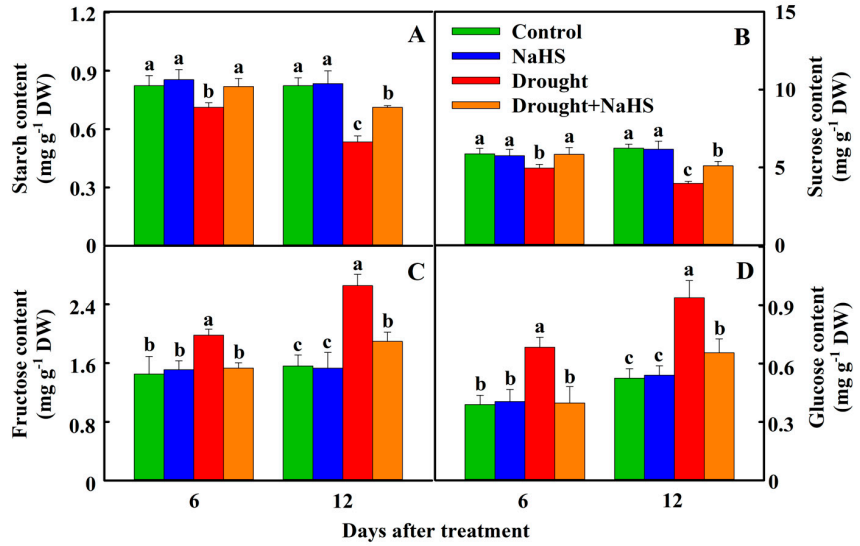

Figure 5. Effects of NaHS on the starch content (**A**), sucrose content (**B**), fructose content (**C**), and glucose content (**D**) of foxtail millet leaves at 6 and 12 days under drought stress. Data are mean ± s.e. (n = 3). Values represented by distinct letters exhibit statistically significant differences ($p < 0.05$).

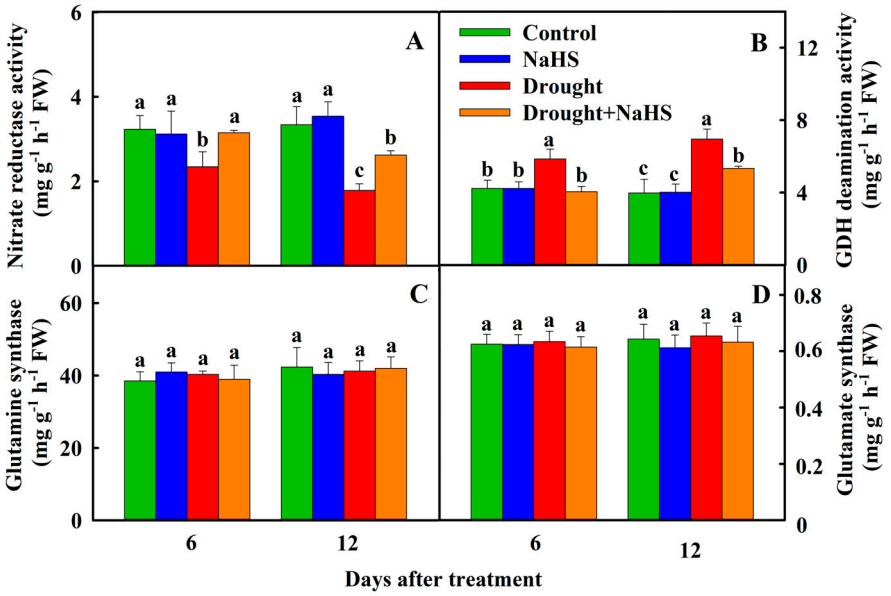

Figure 6. Effects of NaHS on nitrate reductase (NR) activity (**A**), glutamic dehydrogenation (GDH) activity (**B**), glutamine synthase (GS) activity (**C**), and glutamate synthase (GOGAT) activity (**D**) of foxtail millet leaves at 6 and 12 days under drought stress. Data are mean ± s.e. (n = 3). Values represented by distinct letters exhibit statistically significant differences ($p < 0.05$).

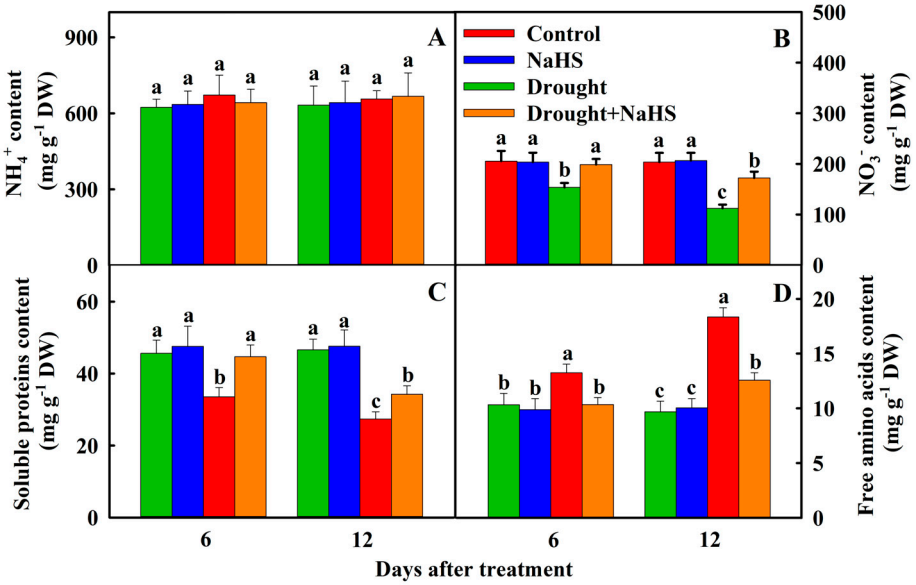

Figure 7. Effects of NaHS on NH_4^+ content (**A**), NO_3^- content (**B**), Soluble protein content (**C**), Free amino acids content (**D**) of foxtail millet leaves at 6 and 12 days under drought stress. Data are mean ± s.e. (n = 3). Values represented by distinct letters exhibit statistically significant differences ($p < 0.05$).

3.5. Effects of Exogenous NaHS on the Expression of Genes Related to Carbon and Nitrogen Metabolism in Foxtail Millet under Drought Stress

The expression of genes encoding the key enzyme *Rubisco* (*Sita101774958*, *Sita101770259*, *Sita101764983*) for carbon fixation was significantly increased by H_2S in foxtail leaves. Furthermore, the transcription levels of sucrose and starch biosynthesis genes *SPS* (*Sita101759319*, *Sita101781780*, *Sita101752535*), *AGPase* (*Sita101771800*), *GBSS* (*Sita101786779*), and *SS* (*Sita101758779*, *Sita101785561*, *Sita101761472*, *Sita101774804*) in plants treated with NaHS were significantly higher compared to untreated plants. NaHS also down-regulated genes

associated with sucrose decomposition, such as *SuSy* (*Sita101762615*, *Sita101783222*) and *INV* (*Sita101774900*, *Sita101765478*, *Sita101770954*). Additionally, NaHS up-regulated genes involved in nitrogen metabolism, including *NR* (*Sita101760744*, *Sita101773167*) and *GS* (*Sita101772334*, *Sita101764285*), while down-regulating the transcription levels of *GDH* (*Sita101778373*, *Sita101755619*) (Figure 8).

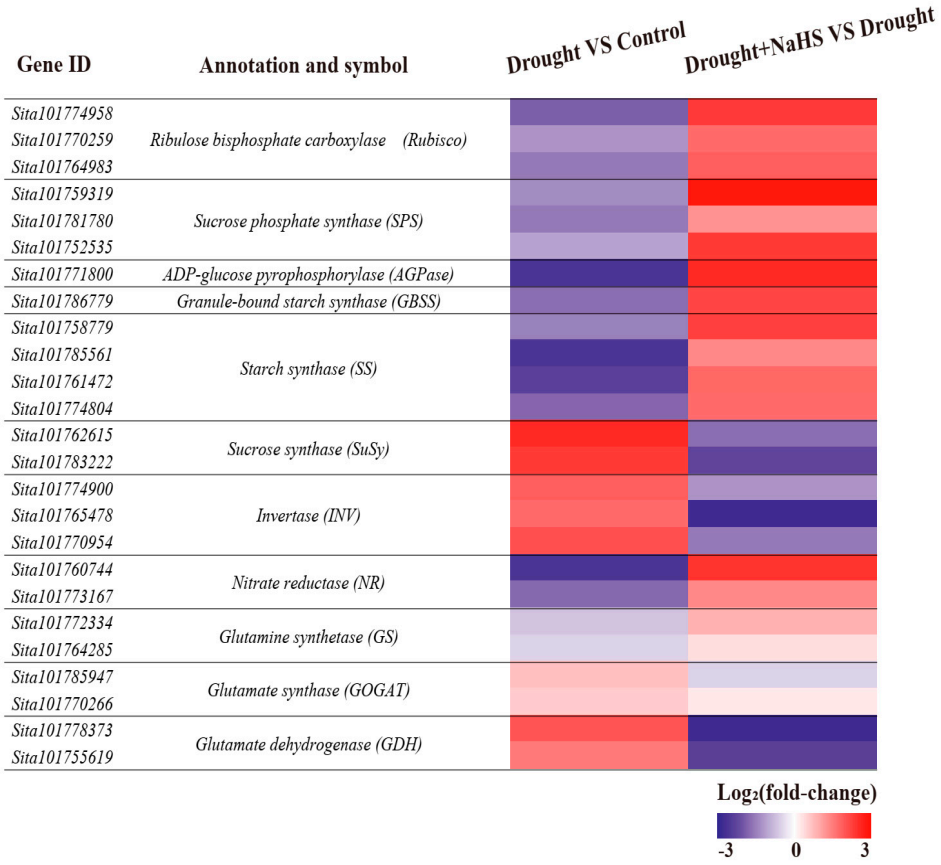

Figure 8. Effects of drought and NaHS on the transcription of genes related to carbon and nitrogen metabolism. The color scale represents the $\log_2$ (fold change) value.

4. Discussion

Drought stress has been shown to hinder plant growth and development, however, the introduction of exogenous H_2S has been found to mitigate the growth inhibition caused by drought [38,39]. The research revealed that water deficiency impeded the growth of foxtail millet, leading to a decreased aboveground dry weight in comparison to non-stressed plants. Conversely, the external administration of NaHS demonstrated a potential alleviation of the suppressive effects of drought stress on foxtail millet growth (Figure 1). This suggests that H_2S may enhance the drought resistance of foxtail millet. Similarly significant increases in above-ground dry weight under drought stress have been reported in safflower pre-treated with H_2S [40], as well as in wheat [41] and peppers [42]. Additionally, research indicates that plants lacking the endogenous H_2S synthesizing gene *DES1* exhibit heightened sensitivity to drought stress [43]. Upon analysis, it was noted that the leaves of dry foxtail millet exhibited a higher H_2S content in comparison to the control group. This observation serves to corroborate the notion that H_2S promotes foxtail millet growth under drought-stress conditions.

Photosynthesis serves as a crucial foundation for crop yield formation, supplying humans with essential sustenance and energy [44,45]. Nevertheless, drought poses a significant threat to this metabolic process in plants. Drought-induced stress diminishes plant photosynthetic efficiency by triggering stomatal closure, disrupting chloroplast integrity, and impeding photosynthetic enzyme function, ultimately impeding plant growth and

yield production [46]. Pn is an important index to evaluate the photosynthetic capacity and growth status of plants. This study demonstrated that foxtail millet treated with NaHS exhibited enhanced Pn and GS in response to drought stress (Figure 2). Similar findings were observed in rice and Chinese cabbage [47,48]. Chlorophyll, a vital pigment molecule located in plant chloroplasts, plays a significant role in the process of photosynthesis. Previous research has elucidated the role of H_2S in spinach photosynthesis, showing that application of NaHS led to increased chlorophyll and soluble protein content, as well as enhanced matrix sheet accumulation and seedling growth [49]. Furthermore, the impact of drought stress on photosynthesis was evident in alterations to photosynthetic electron transport capacity and energy distribution.

Drought-induced stress has been shown to result in a notable reduction in plant leaf area, impairing plant photosynthesis [50,51]. Research has demonstrated that treatment with H_2S can enhance the Fv/Fm in plants experiencing drought stress [52]. By this finding, the foliar administration of NaHS on desiccated foxtail millet foliage results in an increased Fv/Fm, thereby facilitating the utilization of absorbed light energy by PSII in photochemical reactions rather than dissipating it as heat (Figure 3). Carbohydrates, such as sucrose and starch, serve as crucial components for cell growth by acting as structural materials and energy reservoirs in plants [53,54]. The sugar and starch content serve as the standard for assessing carbon assimilation and accumulation. In the absence of drought stress, the application of NaHS has been shown to notably elevate sucrose levels in the leaves of plants [55]. The findings in this study indicate that foxtail millet leaves treated with NaHS exhibited heightened AGPase and SPS activity, starch and sucrose content compared to the control group (Figures 4 and 5). In conclusion, exogenous NaHS can improve the carbon assimilation of foxtail millet by adding the accumulation of photosynthetic products in their leaves under drought stress.

The growth and productivity of plants are significantly influenced by the process of nitrogen metabolism. Water deficiency can decrease enzyme activity involved in nitrogen assimilation, leading to a reduction in nitrogen-containing compounds and impairing nitrogen metabolism. NR activity serves as an indicator of plants' nitrogen use efficiency [56]. Research has demonstrated that H_2S has the potential to enhance NR activity in plants experiencing abiotic stress [57]. In line with these findings, the application of NaHS externally has been shown to significantly elevate NR activity in foxtail millet leaves subjected to drought stress (Figure 6). Plants treated with NaHS exhibited elevated NR activity, yet no discernible disparity in NH_4^+ levels relative to untreated plants (Figure 7). This discrepancy may be attributed to the compensatory mechanism employed by untreated plants in response to nitrogen assimilation inhibition induced by drought stress, wherein GDH activity is heightened. This phenomenon has been previously documented in various plant species, including *Arabidopsis thaliana* under nitrogen deficiency [58] and soybeans under drought stress conditions [59]. The augmentation of ketoglutarate levels facilitated by increased GDH activity enables plants to sustain the tricarboxylic acid cycle and amino acid biosynthesis amidst drought stress [60,61]. Soluble proteins, which are the primary products of plant nitrogen metabolism, are produced through the biosynthesis of amino acids [62]. In response to stressors, plants frequently bolster cell integrity by augmenting the production of osmotic regulatory compounds and modulating osmotic pressure to withstand unfavorable conditions [63–65]. Research indicates that plants enhance protein content to cope with the drought stress conditions, leading to elevated levels of free amino acids [66,67]. Additionally, investigations have demonstrated that treatment with H_2S notably enhances leaf protein content under conditions of drought stress [68]. In this study, the content of soluble protein in the leaves of drought-affected foxtail millet plants treated with NaHS was further increased, and the content of free amino acids was also lower, compared with drought-affected foxtail millet plants not treated with NaHS. Application of exogenous NaHS has a beneficial effect on sustaining metabolic processes and enhancing growth in foxtail millet seedlings under drought-stress conditions. Consequently, NaHS treatment is

shown to positively influence carbon and nitrogen metabolism in drought-stressed foxtail millet plants.

The regulation of carbon and nitrogen metabolism is influenced by genetic factors and environmental conditions [69,70]. In this study, it was observed that drought stress led to a down-regulation of *Rubisco*, *SPS*, *AGPase*, *GBSS*, *SS*, and *NR* genes associated with carbon and nitrogen metabolism in dry foxtail millet leaves compared to the control group. Conversely, the transcription levels of these genes in foxtail millet leaves treated with NaHS were higher than those in plants subjected to drought stress (Figure 8). NaHS treatment was found to enhance the up-regulation of the *GR* gene in foxtail millet, thereby mitigating oxidative damage induced by heavy metal Cr stress [71]. In the presence of nitrogen deficiency, the up-regulation of *Rubisco* gene expression and enhanced nitrogen storage within Rubisco protein were observed in soybean leaves and roots in response to H_2S treatment [59]. Drought stress destroyed the equilibrium of carbon and nitrogen metabolism in crops [15,57]. The increased carbon assimilation observed in NaHS-treated plants was found to be strongly correlated with elevated NO_3^- content and NR activity. Furthermore, under conditions of drought stress, plants treated with NaHS demonstrated the ability to sustain a consistent pattern of carbon and nitrogen metabolism, thereby confirming the principle of coordinated regulation of carbon and nitrogen metabolism. All these findings suggest that H_2S enhances the drought resistance of foxtail millet by regulating carbon and nitrogen metabolism (Figure 9).

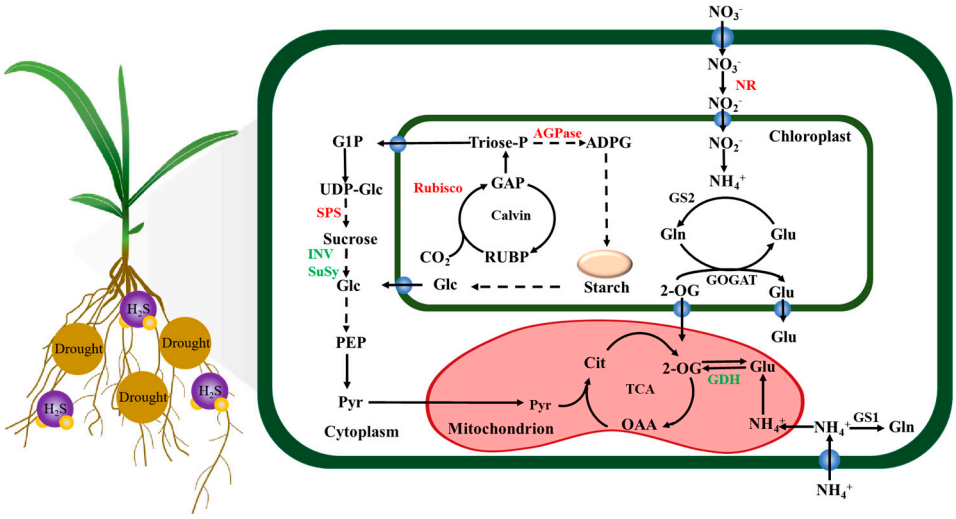

Figure 9. Effects of NaHS on carbon and nitrogen metabolism in foxtail millet leaves under drought. Red and green indicate that the enzyme activities were increased or decreased by NaHS. Rubisco: Ribulose-1,5-bisphosphate carboxylase; AGP: ADP-glucose pyrophosphorylase; INV: invertase; SPS: sucrose-phosphate synthase; NR: Nitrate reductase; GOGAT: glutamic acid synthase activity; GS: glutamine synthetase; GDH: glutamic acid dehydrogenase activity.

5. Conclusions

Exogenous H_2S improved drought tolerance in foxtail millet by modulating carbon and nitrogen metabolism. Specifically, plants treated with NaHS exhibit reduced growth inhibition and decreased photosynthetic capacity under drought stress conditions, attributed to the maintenance of higher growth characteristics, photosynthetic enzyme activity, and energy conversion efficiency. Furthermore, exogenous H_2S treatment has been shown to increase carbohydrate metabolism, ADPGase activity, NO_3^- content, NR activity, and protein content in foxtail millet subjected to drought stress. In summary, this research elucidated the physiological mechanism by which H_2S mitigates drought-induced stress in foxtail millet through the maintenance of carbon and nitrogen metabolism homeostasis.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy14051080/s1, Table S1: Gene-specific primers used in this study; Table S2: Composition and dosage of qRT-PCR reaction system; Table S3: Program used for qRT-PCR.

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References

1. Zhou, Y.; Yang, P.; Cui, F.L.; Zhang, F.T.; Luo, X.D.; Xie, J.K. Transcriptome analysis of salt stress responsiveness in the seedlings of dongxiang wild rice (*Oryza rufipogon* Griff.). *PLoS ONE* **2016**, *11*, e0146242. [CrossRef] [PubMed]
2. Rudack, K.; Seddig, S.; Sprenger, H.; Köhl, K.; Uptmoor, R.; Ordon, F. Drought stress-induced changes in starch yield and physiological traits in potato. *J. Agron. Crop Sci.* **2017**, *203*, 494–505. [CrossRef]
3. Omena-Garcia, R.P.; Martins, A.O.; Medeiros, D.B.; Vallarino, J.G.; Ribeiro, D.M.; Fernie, A.R.; Araújo, W.L.; Nunes-Nesi, A. Growth and metabolic adjustments in response to gibberellin deficiency in drought stressed tomato plants. *Environ. Exp. Bot.* **2019**, *159*, 95–107. [CrossRef]
4. De Roo, L.; Salomón, R.L.; Oleksyn, J.; Steppe, K. Woody tissue photosynthesis delays drought stress in *Populus tremula* trees and maintains starch reserves in branch xylem tissues. *New Phytol.* **2020**, *228*, 70–81. [CrossRef] [PubMed]
5. Zhou, M.; Zhou, H.; Shen, J.; Zhang, Z.; Gotor, C.; Romero, L.C.; Yuan, X.X.; Xie, Y. H_2S action in plant life cycle. *Plant Growth Regul.* **2021**, *94*, 1–9. [CrossRef]
6. Papanatsiou, M.; Scuffi, D.; Blatt, M.; Garcia-Mata, C. Hydrogen sulfide regulates inward-rectifying K^+ channels in conjunction with stomatal closure. *Plant Physiol.* **2015**, *168*, 29–35. [CrossRef] [PubMed]
7. Parveen, M.; Asaeda, T.; Rashid, M. Hydrogen sulfide induced growth, photosynthesis and biochemical responses in three submerged macrophytes. *Flora* **2017**, *230*, 1–11. [CrossRef]
8. Liu, F.J.; Fu, X.; Wu, G.X.; Feng, Y.Q.; Li, F.D.; Bi, H.G.; Ai, X.Z. Hydrogen peroxide is involved in hydrogen sulfide-induced carbon assimilation and photoprotection in cucumber seedlings. *Environ. Exp. Bot.* **2020**, *175*, 104052. [CrossRef]
9. Ali, S.; Nawaz, A.; Ejaz, S.; Haider, S.T.A.; Alam, M.W.; Javed, H.U. Effects of hydrogen sulfide on postharvest physiology of fruits and vegetables: An overview. *Sci. Hortic-Amst.* **2019**, *243*, 290–299. [CrossRef]
10. Guan, M.Y.; Zhang, H.H.; Pan, W.; Jin, C.W.; Lin, X.Y. Sulfide alleviates cadmium toxicity in Arabidopsis plants by altering the chemical form and the subcellular distribution of cadmium. *Sci. Total Environ.* **2018**, *627*, 663–670. [CrossRef]
11. Jin, Z.P.; Wang, Z.Q.; Ma, Q.X.; Sun, L.M.; Zhang, L.P.; Liu, Z.Q.; Liu, D.M.; Hao, X.F.; Pei, Y.X. Hydrogen sulfide mediates ion fluxes inducing stomatal closure in response to drought stress in *Arabidopsis thaliana*. *Plant Soil* **2017**, *419*, 141–152. [CrossRef]
12. Chen, S.S.; Jia, H.L.; Wang, X.F.; Shi, C.; Wang, X.; Ma, P.Y.; Wang, J.; Ren, M.J.; Li, J.S. Hydrogen Sulfide Positively Regulates Abscisic Acid Signaling through Persulfidation of SnRK2.6 in Guard Cells. *Mol. Plant* **2020**, *13*, 732–744. [CrossRef] [PubMed]
13. Wang, L.; Wan, R.; Shi, Y.; Xue, S. Hydrogen Sulfide Activates S-Type Anion Channel via OST1 and Ca^{2+} Modules. *Mol. Plant* **2016**, *9*, 489–491. [CrossRef] [PubMed]
14. Wang, Y.; Wang, Y.M.; Lu, Y.T.; Qiu, Q.L.; Fan, D.M.; Wang, X.C.; Zheng, X.Q. Influence of different nitrogen sources on carbon and nitrogen metabolism and gene expression in tea plants (*Camellia sinensis* L.). *Plant Physiol. Bioch.* **2021**, *167*, 561–566. [CrossRef]
15. Ren, J.H.; Xie, T.; Wang, Y.L.; Li, H.B.; Liu, T.T.; Zhang, S.Q.; Yin, L.N.; Wang, S.W.; Deng, X.P.; Ke, Q.B. Coordinated regulation of carbon and nitrogen assimilation confers drought tolerance in maize (*Zea mays* L.). *Environ. Exp. Bot.* **2020**, *176*, 104086. [CrossRef]
16. Zhou, H.; Chen, Y.; Zhai, F.C.; Zhang, J.; Zhang, F.; Yuan, X.X.; Xie, Y.J. Hydrogen sulfide promotes rice drought tolerance via reestablishing redox homeostasis and activation of ABA biosynthesis and signaling. *Plant Physiol. Bioch.* **2020**, *155*, 213–220. [CrossRef] [PubMed]
17. Lu, H.Y.; Zhang, J.P.; Liu, K.B.; Wu, N.Q.; Li, Y.M.; Zhou, K.S.; Ye, M.L.; Zhang, T.Y.; Zhang, H.J.; Yang, X.Y.; et al. Earliest domestication of common millet (*Panicum miliaceum*) in East Asia extended to 10,000 years ago. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 7367–7372. [CrossRef] [PubMed]
18. Jia, G.; Huang, X.; Zhi, H.; Zhao, Y.; Zhao, Q.; Li, W. A haplotypemap of genomic variations and genome-wide association studies of agronomic traits in foxtail millet (*Setaria italica*). *Nat. Genet.* **2013**, *45*, 957–961. [CrossRef] [PubMed]

19. Numan, M.; Serba, D.D.; Ligaba-Osena, A. Alternative Strategies for Multi-Stress Tolerance and Yield Improvement in Millets. *Genes* **2021**, *12*, 739. [CrossRef]
20. Thakur, M.; Anand, A. Hydrogen sulfide: An emerging signaling molecule regulating drought stress response in plants. *Physiol. Plant.* **2021**, *172*, 1227–1243. [CrossRef] [PubMed]
21. Tian, B.; Qiao, Z.; Zhang, L.; Li, H.; Pei, Y. Hydrogen sulfide and proline cooperate to alleviate cadmium stress in foxtail millet seedings. *Plant Physiol. Bioch.* **2016**, *109*, 293–299. [CrossRef]
22. Prathap, V.; Ali, K.; Singh, A.; Vishwakarma, C.; Krishnan, V.; Chinnusamy, V.; Tyagi, A. Starch accumulation in rice grains subjected to drought during grain filling stage. *Plant Physiol. Bioch.* **2019**, *142*, 440–451. [CrossRef]
23. Xiao, Y.; Wu, X.; Sun, M.; Peng, F. Hydrogen sulfide alleviates waterlogging-induced damage in peach seedlings via enhancing antioxidative system and inhibiting ethylene synthesis. *Front. Plant Sci.* **2020**, *11*, 696. [CrossRef] [PubMed]
24. Lichtenthaler, H.K. Chlorophylls and carotenoids: Pigments of photosynthetic biomembranes. *Method Enzymol.* **1987**, *148*, 350–382. [CrossRef]
25. Liu, J.R.; Ma, Y.N.; Lv, F.J.; Chen, J.; Zhou, Z.G.; Wang, Y.H. Changes of sucrose metabolism in leaf subtending to cotton boll under cool temperature due to late planting. *Field Crop Res.* **2013**, *144*, 200–211. [CrossRef]
26. Schaffer, A.A.; Petreikov, M. Sucrose-to-starch metabolism in tomato fruit undergoing transient starch accumulation. *Plant Physiol.* **1997**, *113*, 739–746. [CrossRef] [PubMed]
27. Hu, W.; Cao, Y.; Loka, D.A.; Harris-Shultz, K.R.; Reiter, R.J.; Ali, S.; Liu, Y.; Zhou, Z. Exogenous melatonin improves cotton (*Gossypium hirsutum* L.) pollen fertility under drought by regulating carbohydrate metabolism in male tissues. *Plant Physiol. Bioch.* **2020**, *151*, 579–588. [CrossRef] [PubMed]
28. Barro, F.; Fontes, A.G.; Maldonado, J.M. Organic nitrogen content and nitrate and nitrite reductase activities in tritordeum and wheat grown under nitrate or ammonium. *Plant Soil* **1991**, *135*, 251–256. [CrossRef]
29. O'neal, D.; Joy, K.W. Glutamine synthetase of pea leaves. I. purification, stabilization, and pH optima. *Arch. Biochem. Biophys.* **1973**, *159*, 113–122. [CrossRef]
30. Matoh, T.; Takahashi, E. Changes in the activities of ferredoxin-and NADH-glutamate synthase during seedling development of peas. *Planta* **1982**, *154*, 289–294. [CrossRef]
31. Loyola-Vargas, V.M.; de Jimenez, E.S. Differential role of glutamate dehydrogenase in nitrogen metabolism of maize tissues. *Plant Physiol.* **1984**, *76*, 536–540. [CrossRef]
32. Xu, H.F.; Zou, Q.Y.; Yang, G.X.; Jiang, S.H.; Fang, H.C.; Wang, Y.C.; Zhang, J.; Zhang, Z.Y.; Wang, N.; Chen, X. MdMYB6 regulates anthocyanin formation in apple both through direct inhibition of the biosynthesis pathway and through substrate removal. *Hortic. Res-Engl.* **2020**, *7*, 72. [CrossRef]
33. Hansen, J.; Moller, I.B. Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. *Analy. Biochem.* **1975**, *68*, 87–94. [CrossRef] [PubMed]
34. Hajlaoui, H.; El Ayeb, N.; Garrec, J.P.; Denden, M. Differential effects of salt stress on osmotic adjustment and solutes allocation on the basis of root and leaf tissue senescence of two silage maize (*Zea mays* L.) varieties. *Ind. Crop Prod.* **2010**, *31*, 122–130. [CrossRef]
35. Zhou, H.; Zhang, J.; Shen, J.; Zhou, M.J.; Yuan, X.X.; Xie, Y.J. Redox-based protein persulfidation in guard cell ABA signaling. *Plant Signal. Behav.* **2020**, *15*, 1741987. [CrossRef]
36. Bräutigam, A.; Gagneul, D.; Weber, A.P. High-throughput colorimetric method for the parallel assay of glyoxylic acid and ammonium in a single extract. *Anal. Biochem.* **2007**, *362*, 151–153. [CrossRef] [PubMed]
37. Patterson, K.; Cakmak, T.; Cooper, A.; Lager, I.; Rasmusson, A.G.; Escobar, M.A. Distinct signalling pathways and transcriptome response signatures differentiate ammonium-and nitrate-supplied plants. *Plant Cell Environ.* **2010**, *33*, 1486–1501. [CrossRef] [PubMed]
38. Huang, D.J.; Huo, J.Q.; Liao, W.B. Hydrogen sulfide: Roles in plant abiotic stress response and crosstalk with other signals. *Plant Sci.* **2021**, *302*, 110733. [CrossRef] [PubMed]
39. Zhang, J.; Zhou, M.J.; Zhou, H.; Zhao, D.D.; Gotor, C.; Romero, L.C.; Shen, J.; Ge, Z.L.; Zhang, Z.R.; Shen, W.B.; et al. Hydrogen sulfide, a signaling molecule in plant stress responses. *J. Integr. Plant Biol.* **2021**, *63*, 146–160. [CrossRef]
40. Amir, S.B.; Rasheed, R.; Ashraf, M.A.; Hussain, I.; Iqbal, M. Hydrogen sulfide mediates defense response in safflower by regulating secondary metabolism, oxidative defense and elemental uptake under drought. *Physiol. Plant.* **2020**, *172*, 795–808. [CrossRef]
41. Ma, D.; Ding, H.; Wang, C.; Qin, H.; Han, Q.; Hou, J.; Lu, H.; Xie, Y.; Guo, T. Alleviation of drought stress by hydrogen sulfide is partially related to the Abscisic Acid signaling pathway in Wheat. *PLoS ONE* **2016**, *11*, e0163082. [CrossRef]
42. Kaya, C.; Shabala, S. Sodium hydrosulfide-mediated upregulation of nitrogen metabolism improves drought stress tolerance in pepper plants. *Environ. Exp. Bot.* **2023**, *209*, 105305. [CrossRef]
43. Jin, Z.; Sun, L.; Yang, G.; Pei, Y. Hydrogen Sulfide Regulates Energy Production to Delay Leaf Senescence Induced by Drought Stress in *Arabidopsis*. *Front. Plant Sci.* **2018**, *9*, 405332. [CrossRef] [PubMed]
44. Martínez-Vilalta, J.; Garcia-Forner, N. Water potential regulation, stomatal behaviour and hydraulic transport under drought: Deconstructing the iso/anisohydric concept. *Plant Cell Environ.* **2017**, *40*, 962–976. [CrossRef]
45. Pilon, C.; Snider, J.L.; Sobolev, V.; Chastain, D.R.; Sorensen, R.B.; Meeks, C.D.; Massa, A.N.; Walk, T.; Singh, B.; Earl, H.J. Assessing stomatal and non-stomatal limitations to carbon assimilation under progressive drought in peanut (*Arachis hypogaea* L.). *J. Plant Physiol.* **2018**, *231*, 124–134. [CrossRef] [PubMed]

46. Yarkhunova, Y.; Guadagno, C.R.; Rubin, M.J.; Davis, S.J.; Ewers, B.E.; Weinig, C. Circadian rhythms are associated with variation in photosystem II function and photoprotective mechanisms. *Plant Cell Environ.* **2018**, *41*, 2518–2529. [CrossRef] [PubMed]
47. Duan, B.B.; Ma, Y.H.; Jiang, M.R.; 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.* **2015**, *75*, 33–44. [CrossRef]
48. Zhang, W.Z.; Wang, L.; Zhang, L.P.; Kong, X.Q.; Zhang, J.; Wang, X.; Pei, Y.X.; Jin, Z.P. H_2S-mediated balance regulation of stomatal and non-stomatal factors responding to drought stress in Chinese cabbage. *Hortic. Res.* **2023**, *10*, uhac284. [CrossRef] [PubMed]
49. Chen, J.; Wu, F.H.; Wang, W.H.; Zheng, C.J.; Lin, G.H.; Dong, X.J.; He, J.X.; Pei, Z.M.; Zheng, H.L. Hydrogen sulphide enhances photosynthesis through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification in Spinacia oleracea seedlings. *J. Exp. Bot.* **2011**, *62*, 4481–4493. [CrossRef] [PubMed]
50. Huang, B.; Chen, Y.E.; Zhao, Y.Q.; Ding, C.B.; Liao, J.Q.; Hu, C.; Zhou, L.J.; Zhang, Z.W.; Yuan, S.; Yuan, M. Exogenous melatonin alleviates oxidative damages and protects photosystem II in maize seedlings under drought stress. *Front. Plant Sci.* **2019**, *10*, 677. [CrossRef]
51. Xu, W.Z.; Deng, X.P.; Xu, B.C.; Gao, Z.J.; Ding, W.L. Photosynthetic activity and efficiency of *Bothriochloa ischaemum* and *Lespedeza davurica* in mixtures across growth periods under water stress. *Acta Physiol. Plant* **2014**, *36*, 1033–1044. [CrossRef]
52. Chen, J.; Shang, Y.T.; Wang, W.H.; Chen, X.Y.; He, E.M.; Zheng, H.L.; Shangguan, Z.P. Hydrogen sulfide-mediated polyamines and sugar changes are involved in hydrogen sulfide-induced drought tolerance in *Spinacia oleracea* seedlings. *Front. Plant Sci.* **2016**, *7*, 1173. [CrossRef]
53. Kaur, H.; Manna, M.; Thakur, T.; Gautam, V.; Salvi, P. Imperative role of sugar signaling and transport during drought stress responses in plants. *Physiol. Plantarum.* **2021**, *171*, 833–848. [CrossRef] [PubMed]
54. McCarthy, J.K.; Smith, S.R.; McCrow, J.P.; Tan, M.; Zheng, H.; Beeri, K.; Roth, R.; Lichtle, C.; Goodenoug, U.; Bowler, C.P.; et al. Nitrate reductase knockout uncouples nitrate transport from nitrate assimilation and drives repartitioning of carbon flux in a model pennate diatom. *Plant Cell* **2017**, *29*, 2047–2070. [CrossRef]
55. Tsuji, C.; Dannoura, M.; Desalme, D.; Angeli, N.; Takanashi, S.; Kominami, Y.; Epron, D. Drought affects the fate of non-structural carbohydrates in hinoki cypress. *Tree Physiol.* **2022**, *42*, 784–796. [CrossRef] [PubMed]
56. Siddiqui, M.H.; Alamri, S.; Mukherjee, S.; Al-Amri, A.A.; Alsubaie, Q.D.; Al-Munqedhi, B.M.A.; Ali, H.M.; Kalaji, H.M.; Fahad, S.; Rajput, V.D.; et al. Molybdenum and hydrogen sulfide synergistically mitigate arsenic toxicity by modulating defense system, nitrogen and cysteine assimilation in faba bean (*Vicia faba* L.) seedlings. *Environ. Pollut.* **2021**, *290*, 117953. [CrossRef] [PubMed]
57. Xie, T.L.; Gu, W.R.; Wang, M.Q.; Zhang, L.G.; Li, C.F.; Li, C.F.; Li, W.H.; Li, L.J.; Wei, S. Exogenous 2-(3, 4-Dichlorophenoxy) triethylamine ameliorates the soil drought effect on nitrogen metabolism in maize during the pre-female inflorescence emergence stage. *BMC Plant Biol.* **2019**, *19*, 107. [CrossRef] [PubMed]
58. Mekonnen, D.W.; Flügge, U.I.; Ludewig, F. Gamma-aminobutyric acid depletion affects stomata closure and drought tolerance of *Arabidopsis thaliana*. *Plant Sci.* **2016**, *245*, 25–34. [CrossRef] [PubMed]
59. Zhang, N.N.; Zou, H.; Lin, X.Y.; Pan, Q.; Zhang, W.Q.; Zhang, J.H.; Wei, G.H.; Shangguan, Z.P.; Chen, J. Hydrogen sulfide and rhizobia synergistically regulate nitrogen (N) assimilation and remobilization during N deficiency-induced senescence in soybean. *Plant Cell Environ.* **2020**, *43*, 1130–1147. [CrossRef] [PubMed]
60. Liu, C.G.; Wang, Y.J.; Pan, K.W.; Zhu, T.T.; Li, W.; Zhang, L. Carbon and nitrogen metabolism in leaves and roots of dwarf bamboo (*Fargesia denudata* Yi) subjected to drought for two consecutive years during sprouting period. *J. Plant Growth Regul.* **2014**, *33*, 243–255. [CrossRef]
61. Tian, J.; Pang, Y.; Zhao, Z. Drought, salinity, and low nitrogen differentially affect the growth and nitrogen metabolism of *Sophora japonica* (L.) in a semi-hydroponic phenotyping platform. *Front. Plant Sci.* **2021**, *12*, 715456. [CrossRef] [PubMed]
62. Georgiadou, E.C.; Kowalska, E.; Patla, K.; Kulbat, K.; Smolińska, B.; Leszczyńska, J.; Fotopoulos, V. Influence of heavy metals (Ni, Cu, and Zn) on nitro-oxidative stress responses, proteome regulation and allergen production in basil (Ocimum basilicum L.). *Plants Front. Plant Sci.* **2018**, *9*, 374129. [CrossRef]
63. Blum, A. Osmotic adjustment is a prime drought stress adaptive engine in support of plant production. *Plant Cell Environ.* **2017**, *40*, 4–10. [CrossRef]
64. Shah, T.M.; Imran, M.; Atta, B.M.; Ashraf, M.Y.; Hameed, A.; Waqar, I.; Shafiq, M.; Hussain, K.; Naveed, M.; Aslam, M.; et al. Selection and screening of drought tolerant high yielding chickpea genotypes based on physio-biochemical indices and multi-environmental yield trials. *BMC Plant Biol.* **2020**, *20*, 171. [CrossRef] [PubMed]
65. Zheng, H.F.; Zhang, X.; Ma, W.J.; Song, J.Y.; Rahman, S.U.; Wang, J.H.; Zhang, Y. Morphological and physiological responses to cyclic drought in two contrasting genotypes of *Catalpa bungei*. *Environ. Exp. Bot.* **2017**, *138*, 77–87. [CrossRef]
66. Ozturk, M.; Turkyilmaz Unal, B.; García-Caparrós, P.; Khursheed, A.; Gul, A.; Hasanuzzaman, M. Osmoregulation and its actions during the drought stress in plants. *Physiol. Plantarum.* **2021**, *172*, 1321–1335. [CrossRef] [PubMed]
67. Coruzzi, G.M.; Zhou, L. Carbon and nitrogen sensing and signaling in plants: Emerging 'matrix effects'. *Curr. Opin. Plant Biol.* **2001**, *4*, 247–253. [CrossRef] [PubMed]
68. Xuan, L.; Li, J.; Wang, X.; Wang, C. Crosstalk between hydrogen sulfide and other signal molecules regulates plant growth and development. *Int. J. Mol. Sci.* **2020**, *21*, 4593. [CrossRef] [PubMed]

69. Masclaux-Daubresse, C.; Daniel-Vedele, F.; Dechorgnat, J.; Chardon, F.; Gaufichon, L.; Suzuki, A. Nitrogen uptake, assimilation and remobilization in plants: Challenges for sustainable and productive agriculture. *Ann. Bot* **2010**, *105*, 1141–1157. [CrossRef] [PubMed]
70. Zhang, C.C.; Zhou, C.Z.; Burnap, R.L.; Peng, L. Carbon/Nitrogen Metabolic Balance: Lessons from Cyanobacteria. *Trends Plant Sci.* **2018**, *23*, 1116–1130. [CrossRef]
71. 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* **2014**, *56*, 472–481. [CrossRef]