Interactions between hydrogen sulfide and rhizobia modulate the physiology and metabolism during water deficiency-induced oxidative defense in soybean

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Abstract

Hydrogen sulphide (H2S), as a new gas signal molecule, participates in the regulation of a variety of abiotic stresses in plants. However, it was unclear how H2S and rhizobia can together to affect the adaptation of soybean to water deficiency. Here, the adaptation mechanism of H2S and rhizobia in soybean to water deficiency was studied. Our results showed that H2S and rhizobia jointly enhanced leaf chlorophyll content, the relative water content (RWC) and caused an increase biomass in soybean under water deficiency. Besides, under water deficiency, H2S enhanced biomass by affecting nodule numbers and nitrogenase activity during the growth of soybean. The expression of soybean nodulation marker genes including early nodulin 40 (GmENOD40), ERF required for nodulation (GmERN), and nodulation inception genes were up-regulated by H2S and rhizobia in nodules. Moreover, the combined effect of H2S and rhizobia were proved to affect the enzyme activities and gene expression level of antioxidant, as well as osmotic protective substance under water deficiency. In addition, the metabolomics results provided that the changes of lipids and lipid-like molecules were remarkably promoted by the combined effect of H2S and rhizobia. Thus, H2S and rhizobia synergistically subsided the oxidative damage by increasing the accumulation of metabolites and strengthening the antioxidant capacity under water deficiency.

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Abstract

Hydrogen sulphide (H2S), as a new gas signal molecule, participates in the regulation of a variety of abiotic stresses in plants. However, it was unclear how H2S and rhizobia can together to affect the adaptation of soybean to water deficiency. Here, the adaptation mechanism of H2S and rhizobia in soybean to water deficiency was studied. Our results showed that H2S and rhizobia jointly enhanced leaf chlorophyll content, the relative water content (RWC) and caused an increase biomass in soybean under water deficiency. Besides, under water deficiency, H2S enhanced biomass by affecting nodule numbers and nitrogenase activity during the growth of soybean. The expression of soybean nodulation marker genes including early nodulin 40 (GmENOD40), ERF required for nodulation (GmERN), and nodulation inception genes were up-regulated by H2S and rhizobia in nodules. Moreover, the combined effect of H2S and rhizobia were proved to affect the enzyme activities and gene expression level of antioxidant, as well as osmotic protective substance under water deficiency. In addition, the metabolomics results provided that the changes of lipids and lipid-like molecules were remarkably promoted by the combined effect of H2S and rhizobia. Thus, H2S and rhizobia synergistically subsided the oxidative damage by increasing the accumulation of metabolites and strengthening the antioxidant capacity under water deficiency.

Keywords: Hydrogen sulphide (H_2S), Rhizobia, Soybean (G*lycine max*), Antioxidant defense, Osmotic adjustment, Metabolomics

Introduction

All life activities of plants are carried out with the participation of water, and sufficient water is an important condition for plant growth and development (Gupta et al., 2020). Water deficiency has negative effects on plant growth and development, which further reduces plant production (Ashraf, 2010; Parvin et al., 2019). These negative effects are mainly due to the imbalance of water metabolism and oxidative damage induced by water deficiency (Amrutha et al., 2019). A significant change in plant growth, photosynthesis, biomass production, enzymatic activity, and oxidative damage parameters has been found in plants under drought conditions (Aref et al., 2013; Cotado et al., 2020; Husen et al., 2017; Matos et al., 2010). Therefore, understanding the water deficiency mechanisms of soybean is indispensable in developing drought resistant variety to assure sustainable soybean production in water-deficiency regions.

Water deficiency breaks the balance between the production and removal of reactive oxygen species (ROS) in plants. Plants manifest an excessive increment in ROS generation in response to water deficiency (Miller et al., 2010). Enhanced ROS generation under drought produces substantial damages to the cellular components, which causes membrane injuries, oxidative stress, protein degradation, and enzyme inactivation (Faize et al., 2011; Hajiboland et al., 2017; Munné-Bosch & Peñuelas, 2003). To fight against water stress-induced oxidative damage, plants have an efficient antioxidant defense system, which includes enzymatic system and non-enzymatic system (Ashraf et al., 2015; Sharma et al., 2012). Efficient enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) play critical roles in removing the water

deficiency-induced excessive ROS (Anjum et al., 2017). In plants, ascorbate-glutathione (AsA-GSH) cycle is a very crucial component of the antioxidant defense system (Hasanuzzaman et al., 2017; Zhang et al., 2013). The AsA-GSH cycle is depended on the activities of ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR). DHAR and MDHAR realize the regeneration of reduced ascorbate (AsA) in this cycle. GR is responsible for the regeneration of reduced glutathione (GSH) (Shan et al., 2018). Moreover, plants synthesize different permeates or solutes through metabolic activities to maintain water balance under adversity conditions (Krasensky & Jonak, 2012). For instance, previous studies showed the accumulation of proline and soluble sugars increase the ROS elimination and maintain the growth of peppermint (*Mentha piperita*) and *Catharanthus roseus* under water deficiency conditions (Alhaithloulet al., 2019). In addition, high levels of glycinebetaine (GB) and soluble sugars significantly enhance water deficiency tolerance in *Spinacia oleracea* seedlings (Chen et al., 2016).

Legumes are natural hosts of rhizobia, converting nitrogen in the atmosphere into nitrogen available to plants, thus improving the ability of nitrogen-fixing in plants (Janet & Sprent, 1972; Li et al., 2019; Vitousek et al., 2002). Numerous studies have confirmed that legume symbiosis can effectively improve the adaptation of plant to environmental stress (Furlan et al., 2017; Liu et al., 2019). For example, rhizobia can promote plant tolerance to salt stress by increasing antioxidant enzyme activities, osmoregulation capacities, and flavonoid metabolism in soybean and alfalfa (Meng et al., 2016; Qu et al., 2016; Wang et al., 2016). Alternatively, rhizobia enhanced the low temperature tolerance of plants by affecting N metabolism and N absorption (Zhang et al., 2003). Moreover, we recently demonstrated that rhizobia reinforced the Cu tolerance by increasing the activities of antioxidant and the AsA-GSH enzymes in the soybean seedlings (Chen et al., 2018). However, how the soybean-rhizobia symbiotic system responds to water deficiency and the specific mechanism are still unclear.

Hydrogen sulphide (H_2S), as an emerging gas signal molecule, together with nitric oxide (NO) and carbon monoxide (CO), plays an important role in plant growth and development (Li et al., 2013; Li et al., 2016; Luo et al., 2020; Wang et al., 2012). For instance, H_2S can promote seed germination and root development in *Arabidopsis* (Baudouin et al., 2016), participate in the stomatal movement by mediating 8-mercaptocyclic GMP in*Arabidopsis* (Honda et al., 2015). Chen et al. (2011) reported that H_2S promotes photosynthesis by increasing ribulose-1, 5-bisphosphate carboxylase activity and thiol redox modification in *S. oleracea* . Additionally, H_2S regulates pepper's antioxidant system to alleviate zinc toxicity (Kaya et al., 2018), and enhances the drought tolerance through effecting on the biosynthesis of GB and soluble sugars in *S. oleracea*seedlings (Chen et al., 2016). In our previous study, it was found that H_2S alleviates iron deficiency by promoting iron availability and plant hormone levels in *Glycine max* seedlings (Chen et al., 2020). Additionally, H_2S can enhance the establishment of the soybean-rhizobia symbiotic nitrogen fixation system (Zou et al., 2020; Zou et al., 2019). In addition, we recently proved that under nitrogen deficiency condition, the interaction of H_2S and rhizobia increase biomass and yield, improve the nitrogen utilization efficiency, and affect the expression of senescence related genes in soybean plants (Zhang et al., 2020). However, it is not clear whether H_2S and rhizobia participate in the regulation the adaptation of soybean plants to water deficiency environment.

The aim of this study was to investigate the antioxidant role of H_2S and rhizobia in soybean under water deficiency, and the mechanism of how they together regulate the osmoprotectants and differential metabolites change in response to water deficiency. In this present study, we found that H_2S and rhizobia can improve against oxidative damage of soybean to water deficiency through regulating symbiotic nodulation, antioxidant system, and osmoprotectants, thus enhancing the photosynthesis and the drought tolerance of plants. Moreover, based on non-target metabolomics methods, we explored that H_2S and rhizobia may enhance water deficiency tolerance by changing the lipid-related metabolites compounds in soybean leaves under water deficiency condition. Together, it was proved that rhizobia symbiosis and H_2S can jointly improve the adaptability of water deficiency in soybean.

Materials and methods

Plant growth and Treatment

Soybean seeds (*Glycine max* , Zhonghuang 13) were sterilized by 75% ethanol for 30 seconds and 50% sodium hypochlorite for 4 min, and washed with distilled water, and then placed on a 1% agar plate for 72 h to germination in a 28°C incubator. After 3 days of culture, the soybean seedlings were selected and cultured into growth medium with nutrient solution (vermiculite and perlite, v/v = 2:1; nitrogen-free nutrient solution 100 mg/L $CaCl_2$, 100 mg/L KH_2PO_4, 50 mg/L Ferric citrate, 150 mg/L NaH_2PO_4, 120 mg/L $MgSO_4 \cdot 7H_2O$, 2.86 mg/L H_3BO_3, 2.3 mg/L $MnSO_4 \cdot 4H_2O$, 2.8 mg/L $ZnSO_4 \cdot {}_7H_2O$, 13 mg/L $Na_2MoO_4 \cdot 2H_2O$, 2.2 mg/L $CaSO_4 \cdot 5H_2O$). The plants were grown in a controlled growth chamber with a light/dark state of 10/14 h, relative humidity of 80%, temperature of 23/25°C, and photosynthetically active radiation of 190 μmol $m^{-2}s^{-1}$.

The 7-day-old seedlings were divided into four treatment groups: Control, without rhizobia and NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation (*Sinorhizobium fredii* Q8 strain); Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. In addition, we set three water gradients again for each group: normal water content 80%-90% (NW), moderate drought water content 50%-60% (MW), severe drought water content 20%-30% (SW). When the first true leave of soybean was fully expanded, all the seedlings of the Q8 and Q8+NaHS treatment groups were inoculated with 10 ml of rhizobia suspension (OD_{600}= 0.5). Seedlings of the NaHS and Q8+NaHS treatment groups were added with 10 ml of 100 μM NaHS solution every 3 days until harvest. While the other two groups of seedlings were watered with double distilled water. Water contents were controlled with an electronic balance after the soybean seedlings were inoculated with rhizobia and growth for 21 days. The seedling bags were weighed every 1 or 2 days, and distilled water was used to add moisture if it was necessary. Half of the samples were dried to constant weight for dry matter determine, and the other half was immediately frozen in liquid nitrogen and stored at -80°C.

Chlorophyll content and leaf RWC measurements

Chlorophyll content was measured using a SPAD-502PLUS chlorophyll meter (Konica Minolta, Kumamoto, Japan), and the leaves (the top fully expanded leaves) were measured at 10:00 a.m.

The relative water content (RWC) of leaf samples was determined as described by Yoo et al. (2010). RWC was calculated using the equation: RWC (%) = (Fresh weight - Dry weight/Turgid weight - Dry weight) x100.

Determination of nitrogenase activity in nodules

Nitrogenase activity was quantified using the acetylene reduction method by Fishbeck et al. (1973) with slight modifications. Fresh soybean nodules (0.2g) were picked and transferred to 25 ml rubber-sealed glass bottles filled with a mixture of acetylene and air (v: v = 1: 100). The bottle was incubated at 28degC for 2 h, and then the ethylene content was measured using a gas chromatograph system (Trace GC Ultra, United States).

Determination of photosynthetic parameters

Photosynthetic rate (Pn), stomatal conductance (Gs), intercellular carbon dioxide concentration (Ci), and transpiration rate (Tr) were measured using a portable photosynthesis system (Li-6400, LiCor, Lincoln, NE, USA) on the second fully expanded leaf of soybean seedling. The WUE was calculated as the ratio of Pn/Tr. Air temperature, effective photosynthetic radiation (PAR), air relative humidity and CO_2 concentration were maintained at 25degC, 800 μmol$\cdot$ $m^{-2} \cdot s^{-1}$, 70%, 400 μmol $\cdot$ mol^{-1}, respectively.

Determination of chlorophyll fluorescence parameters

The PAM-2100 portable modulation fluorometer (PAM 2100, Walz, Germany) was used for measurement. Before measurement, leaves were pretreated in the dark for 30 min. Fo, Fm, Fv (= Fm - Fo), and Fv/Fm parameters were recorded for 15 s at a photon flux density of 4000 μmol $m^{-2} \cdot s^{-1}$. Additionally, we measured the steady-state fluorescence level (Fs') under continuous illumination, the maximal fluorescence level (Fm') induced by a saturating light pulse at the steady-state, and the minimum fluorescence level (Fo') after exposure to far-red light for 3 s. Then, Fv' was calculated using the formula Fv' = Fm'-Fo'. PSII was

calculated using the formula PSII = 1-(Fs'/Fm'). The ETR was calculated using the formula ETR = PARxPSIIx0.85x0.5. NPQ was calculated using the formula NPQ = (Fm/Fm')-1. qP was calculated using the formula qP = (Fm'-Fs)/(Fm'-Fo') (Florian et al., 2009).

H_2S determination

The endogenous H_2S content in root nodules and leaves of soybean was measured using endogenous H_2S assay kit (Comin Biotechnology, Suzhou, China). The absorbance at 665 nm was measured on a spectrophotometer.

Determination of MDA, H_2O_2 and OFR content

To determine malonaldehyde (MDA) content in soybean leaves, we referred to the protocol of Hasanuzzaman et al. (2017) with slight modification. The absorbance at 532 and 600 nm were measured on a spectrophotometer. Hydrogen peroxide (H_2O_2) content was measured according to the method of Hasanuzzaman et al. (2017). The absorbance of the obtained solution was read at 415 nm. The production of an oxygen free radical (OFR) was determined using an OFR reagent kit (Comin Biotechnology, Suzhou, China). OFR scavenging ability was calculated using OD_{530}.

Determination of antioxidant

The activities of superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and ascorbate peroxidase (APX) were measured using enzymatic activity assay kits (Comin Biotechnology, Suzhou, China). The whole procedure was conducted following the instructions of the manufacturer. The SOD, POD, CAT, and APX activities were measured with an EPOCH ultraviolet spectrophotometer (BioTek, Vermont, U.S.A) at OD_{560}, OD_{470}, OD_{240} and OD_{290}, respectively.

Determination of glutathione and ascorbate contents

For measuring the reduced glutathione (GSH) and oxidized glutathione (GSSG) content in soybean leaves, we used a GSH and GSSG assay kit (Comin Biotechnology, Suzhou, China) following the manufacturer's instructions. GSH and GSSG content were measured at OD_{412}. The ascorbic acid (ASA) and dehydroascorbate (DHA) content determination of the soybean leaves were measured the manufacturer's protocols for the ASA and DHA kits (Comin Biotechnology, Suzhou, China). ASA and DHA content were measured at OD_{402} and OD_{265}. The activities of monodehydro reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) were measured using enzymatic activity assay kits (Comin Biotechnology, Suzhou, China) according to the manufacturer's instructions. MDHAR, DHAR, and GR activities were determined at OD_{340}, OD_{265} and OD_{340}, respectively.

Measurement of Glucose, Fructose, Sucrose, PRO and GB content

The content of sucrose, fructose, and glucose were measured using assay kits (Comin Biotechnology, Suzhou, China). Sucrose, fructose, and glucose content were measured at OD_{480}, OD_{480}, and OD_{505}, respectively. The proline (PRO) and glycine betaine (GB) assay kit (Comin Biotechnology, Suzhou, China) was used to measure the PRO and GB content at OD_{520} and OD_{525} in soybean leaves, respectively.

Total RNA isolation, reverse transcription, and gene expression analysis

Total RNA was isolated from frozen plant samples using the TaKaRa MiniBEST Plant RNA Extraction Kit (Takara, Dalian, China) according to the manufacturer's instructions. RNA purity and concentration were determined using an Epoch Microplate spectrophotometer (BioTek, Winooski, VT, USA), whereas the integrity of the RNA was evaluated by 1% agarose gel electrophoresis. Following the manufacturer's recommendations, TaKaRa PrimerScript RT Master Mix (Takara, Dalian, China) was used for reverse transcription and total RNA (1 μg) was used for cDNA synthesis. qRT-PCR was performed using a Quantstudio 6 Flex real-time PCR system (Thermo Fisher, Carlsbad, California, California, USA) and 2x FAST qPCR Master Mixture (with OOX II) (DiNing, Dalian, China). Every qRT-PCR sample contained 1 μl of cDNA, including those for antioxidant related genes, osmoprotectants related genes and symbiosis-related genes (Table S1), 10 μl of EvaGreen 2× qPCR MasterMix (DiNing, Dalian, China), 2 μl of primer and 7 μl of

sterilized water. The qRT-PCR cycle parameters were as follows: 10 min at 95°C, and then 40 cycles of 30 s at 95°C and 1 min at 60°C. Three independent replicates were performed for each sample. The *Gmactin* and*Gm60s* were used as endogenous Control genes were expressed as 2^{-Ct} using the comparative threshold cycle (Ct) method (Livak & Schmittgen, 2001).

Metabolite sample processing

Leaves were randomly collected from different treatments and immediately frozen in liquid nitrogen and stored at -80 °C for metabolite extraction. Frozen leaf tissues (˜10–35 mg) were ground in liquid N using a mortar and pestle to ensure that the samples are metabolically inactive. Frozen powder was homogenized in ice-cold solution of methanol: chloroform: water (3:1:1), with the addition of ribitol (0.2 mg ml^{-1} of methanol) as an internal standard. The slurry was mixed for 5 min using a microtube mixer. Approximately 160 µl of distilled water was added to the extraction solution to separate the polar and nonpolar phases. After centrifugation, only the upper layer (polar phase) was used for further analysis. A quality control (QC) sample was prepared by mixing aliquots from each of the samples.

Gas chromatography-mass spectrometry analysis

After derivatization, samples (1 µl) were injected randomly in splitless mode with a cold injection system (Gerstel, Mülheim a der Ruhr, Germany) into GC (Agilent GC6890, San Jose, CA, USA) and analysed as described previously (Watanabe et al., 2013). The numerical analyses of metabolome were based on the peak height values of the response values. Chromatograms were processed using high throughput data analysis method. These values were normalized by the sample fresh weight and ribitol (internal standard), using the cross-contribution-compensating multiple standard normalization algorithm (Rotaru & Sinclair, 2009).

Data analysis

For assessing plant growth parameters, enzyme activity, and gene expression analysis, statistical significance was used for analysis of variance (One-way ANOVA) in SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Duncan's post-test ($P < 0.05$) was used for multiple comparisons. The results were expressed as the mean +- *SE* . In all figures, different capital letters indicate significant differences between same treatments, under the different water condition, whereas the lowercase letters indicate significant differences between different treatments seedlings under the same water condition. The experiment was carried out with three biological replicates, and each experimental index was measured by at least four replicates.

Results

Effects of H_2S and rhizobia on the shoot and root biomass, chlorophyll content, and leaf relative water content (RWC) under water deficiency condition

As shown in Fig. 1A-C, compared with control, Q8 and Q8+NaHS treatments substantially increased the shoot and root biomass of soybean under the three water conditions. With the decrease of water content, the shoot and root biomass were appreciably reduced by Q8 and Q8+NaHS treatments plants, but were not obviously changed by control and NaHS treatments plants (Fig. 1A-C). Similarly, the chlorophyll content was remarkably increased by Q8 and Q8+NaHS treatments compared with the control and NaHS treatments under the three water conditions, and showed a slight increasing trend in all treatments with the decrease of water content (Fig. 1D). In addition, with the decrease of water content, the RWC of the four treatments had different levels of reduction, but no matter what water content, Q8 and Q8+NaHS treatments were remarkably higher than other two treatments (Fig. 1E).

Effect of H_2S and rhizobia on the number of soybean nodules, endogenous H_2S content and nitrogenase activity under water deficiency condition

Soybean nodules numbers were prominently enhanced by Q8 and Q8+NaHS treatments under MW condition compared with NW and SW conditions, whereas no significant difference was observed between Q8 and Q8+NaHS treatments under MW and SW condition (Fig. 2A, B). Interestingly, the increase in the nodules number did not lead to enhance the root nodule biomass. On the contrary, under MW condition plant

treated with Q8 exhibited a 49.9% loss of nodules biomass compared with the NW condition, and the MW condition plant treated with Q8+NaHS exhibited a 44.2% loss of nodules biomass compared with the NW condition (Fig. 2C). In addition, the endogenous H_2S content of the nodule was reduced by Q8 and NaHS+Q8 treatments with the decrease of water content. Under SW condition, the endogenous H_2S content of the nodule was observably higher by Q8+NaHS treatment than Q8 treatment, but it was no apparent difference between the NW and MW conditions (Fig. 2D). Additionally, the nitrogenase activities were reduced by Q8 and Q8+NaHS treatments with the decrease of water content, similar to the changes of nodule endogenous H_2S (Fig. 2E), but there was a slight enhancement of the nitrogenase activity by Q8+NaHS treatment under the three water conditions compared with Q8 treatment.

Effects of H_2S and rhizobia on photosynthetic parameters of soybean plants under water deficiency

As shown in Fig. 3A and 3D, Pn and Tr were obviously increased by Q8 and Q8+NaHS treatments under the three water conditions. With the decrease of water content, the Pn and Tr of four treatments had a slight downward trend, especially for Q8 and Q8+NaHS treatments, they were significantly decreased under SW condition compared with that the NW condition (Fig. 3A and 3D). With the decrease of water content, the Gs were substantially reduced by Q8 and Q8+NaHS treatments. However, under the same water condition, the Gs were markedly enhanced by Q8 and Q8+NaHS (Fig. 3B). Under same water conditions, the apparent increase of the Ci was observed by Q8 and Q8+NaHS treatments (Fig. 3C). Under same water conditions, Ls was greatly reduced by Q8 and Q8+NaHS treatments compared with that of the control, but no significant difference of the trend of the four treatments was observed under different water conditions (Fig. 3E). In addition, the change trends of WUE were similar to Ls. The difference was that the Q8 and Q8+NaHS treatments displayed a tendency to increase initially and then decrease, but WUE was not markedly changed by control plants (Fig. 3F).

Effects of H_2S and rhizobia on Chlorophyll fluorescence parameters of soybean plant under water deficiency

With the decrease of water content, ETR and PSII displayed a tendency to increase initially and then decrease by control plants. By Q8 treatment, ETR and PSII were straight down with the decrease of water content. However, ETR and PSII were reduced by NaHS and Q8+NaHS treatments under MW condition (Fig. 4A and B). Among the three water conditions, the NPQ was increased in different degrees by Q8 and Q8+NaHS treatments, and the NPQ of control treatment increased with the decrease of water content, but the NPQ did not greatly affect under other treatments (Fig. 4C). Among the three water conditions, Fv/Fm was conspicuously increased by Q8 and Q8+NaHS treatments in soybean leaves. With the decrease of water content, the Q8 and Q8+NaHS treatments partially rescued the decrease of Fv/Fm in soybean leaves (Fig. 4D).

Changes of endogenous H_2S, lipid membrane peroxidation, hydrogen peroxide, and superoxide anion in leaves of soybean plants under water deficiency

The endogenous H_2S contents were reduced by NaHS, Q8 and Q8+NaHS treatments condition under water deficiency (Fig. 5A). Under NW and SW conditions, the endogenous H_2S content of Q8+NaHS treatment was higher than that of control, and the endogenous H_2S content was not markedly regulated by Q8 and Q8+NaHS treatments under MW condition (Fig. 5A). With the decrease of water content, the MDA content and OFR content were obviously increased by four treatments (Fig. 5B and 5D). Under SW condition, membrane lipid peroxidation was increased by 20.5% and 14.7% in Q8 and Q8+NaHS treatments, respectively, compared with control (Fig. 5B). The OFR content was reduced by Q8+NaHS treatment compared with the control under SW condition (Fig. 5D). With the decreases of water content, the H_2O_2 content was not obviously influenced by control treatment. However, the H_2O_2 content of NaHS, Q8 and Q8+NaHS treatments displayed a tendency to increase initially and then reduce with the decrease of water content. In addition, under three water conditions, the H_2O_2 content of the Q8+NaHS treatment was considerably lower than the control (Fig. 5C).

Effect of H_2S and rhizobia on antioxidant enzyme system in soybean plant leaves under water deficiency

As shown in Fig. 6A, with the decrease of water content, the SOD activity showed a trend of increased first and then decreased by control and NaHS treatments, and the SOD activity was decreased in varying degrees by Q8 and Q8+NaHS treatments (Fig. 6A). Under the three water conditions, the POD activity was sharply increased by Q8 and Q8+NaHS treatments. With the decrease of water content, the POD activity was no apparent changed under control treatment, but NaHS treatment showed a trend of increasing first and then decreasing. Moreover, the POD activity was obviously inhibited by Q8+NaHS treatments under MW and SW conditions (Fig. 6B). The CAT activity was substantially enhanced by four treatments under SW condition compared with NW condition. In contrast, the CAT activity was increased by 5.0 and by 5.3-fold under the Q8 and Q8+NaSH treatments compared with control under SW condition, respectively (Fig. 6C). In addition, with the decrease of water content, the APX activity was notably enhanced by control and Q8+NaHS treatments. However, the APX activity of NaHS and Q8 treatments displayed a tendency to decrease initially and then increase, and no matter what the water content, the APX activity by Q8+NaHS treatment was higher than control (Fig. 6D).

Effects of H_2S and rhizobia on AsA-GSH cycle in soybean plants under water deficiency

With the decrease of water content, the GSH content was no apparent increased by Q8 and Q8 + NaHS treatments under MW and SW conditions, and not obvious differences between the Q8 and Q8 +NaHS treatments (Fig. 7A). However, water deficiency caused an obvious change in GSSG content under four treatments. Compared with control, the GSSG content was substantially increased by Q8 and Q8+NaHS treatments under other three water conditions. Moreover, with the decrease of water content, the GSSG content had an obvious increase trend under Q8+NaHS treatment (Fig. 7B). Interestingly, Q8 and Q8+NaHS treatments increased the GSSG content under water deficiency in leaves, the ratio of GSH/GSSG was significantly reduced by two treatments (Fig. 7C).

In addition, the AsA content in control treatment was notably increased under SW condition compared with NW condition. With the decrease of water content, the AsA content by Q8 and Q8+NaHS treatments displayed a tendency to increase initially and then decrease. Under SW condition, the AsA content showed a significant decrease under Q8 and Q8+NaHS treatments compared with control (Fig. 7D). Compared with control, the DHA content was substantially reduced by Q8 and Q8+NaHS treatments under NW and MW conditions, but the change under SW condition was not obvious (Fig. 7E). With the decrease of water content, the DHA content was greatly increased by Q8 and Q8+NaHS treatments, while showed a downward trend under control treatment (Fig. 7E). As a result, we found that under NW and MW conditions, the AsA/DHA ratio of the four treatments had similar changing trends, and the AsA/DHA ratio of Q8 and Q8+NaHS treatments was much higher than that of control treatment. In addition, the AsA/DHA ratio of control treatment was significantly higher than other treatments under SW condition (Fig. 7F).

In addition, the MDHAR activity was affected by water deficiency under the four treatments, and Q8+NaHS treatment promoted MDHAR activity in the leaves under SW condition (Fig. 7G). Interestingly, the DHAR activity of Q8 and Q8+NaHS treatments was appreciably higher than that of control treatment under any water content. With the decrease of water content, the DHAR activity of leaves by the four treatments decreased to varying degrees. (Fig. 7H). The changes of GR activity among the four treatments of SW condition were similar to those of NW condition. The GR activity of Q8 and Q8 +NaHS treatments were much higher than that of control treatment (Fig. 7I).

Effects of H_2S and rhizobia on osmotic adjustment substances of soybean plants under water deficiency

Compared with control, Q8 and Q8+NaHS treatments increased the sucrose content to varying degrees under any water content conditions. With the decrease of water content, the sucrose content of control, Q8 and Q8+NaHS treatments had different degrees of increase (Fig. 8A). Our results showed that the changing trends of fructose and sucrose content were very similar, the fructose content in leaves displayed a tendency to increase with the decreases of water content, which was markedly enhanced by Q8 and Q8+NaHS treatments (Fig. 8B). Glucose content also exhibited a significant increase by Q8 and Q8+NaHS treatments under three water conditions. Meanwhile, with the decrease of water content, excepted for NaHS treatment, the glucose

content showed different increases in the other three treatments (Fig. 8C). In addition, In addition, we also found that the PRO content was increased to varying degrees by the four treatments with the decrease of water content, while the PRO content was sharply increased by Q8 and Q8+NaHS treatments under SW conditions (Fig. 8D). With the decrease of water content, the GB content was elevated by Q8+NaHS treatment, and the GB content of Q8 and Q8+NaHS treatments were higher than that of control treatment under three water conditions (Fig. 8E).

RT-qPCR analysis of antioxidants, sugar synthesis and symbiosis-related gene expression in soybean plants under water deficiency

As shown in the Fig. 9A, the expression level of *GmCAT* was appreciably down-regulated by Q8 and Q8+NaHS treatments under three water conditions. Similarly, the expression abundance of *GmSOD1* was significantly down-regulated by Q8 and Q8+NaHS treatments under any water content conditions (Fig. 9B). On contrast, the expression abundances of *GmSOD2* was greatly up-regulated by 49.3 and 144.1 fold in the Q8 and Q8+NaHS treatment compared with that of the control treatment under SW condition, respectively (Fig. 9C). Additionally, compared with control treatment, the expression levels of *GmPrx* ,*GmGrx* and *GmBADH* were substantially down-regulated in Q8+NaHS treatment under MW condition, but were up-regulated to different degrees under SW condition (Fig. 9E-F). With the decrease of water content, the expression abundance of *GmSUS* was not remarkably different among four treatments. In addition, in the Q8+NaHS treatment, the expression level of *GmSUS* was considerably lower than control treatment under any water content conditions (Fig. 9G). Under three water conditions, the expression abundance of *GmUDP* showed a appreciably down-regulation in Q8+NaHS treatment. With the decrease of water content, NaHS treatment apparently increased the expression abundance of *GmUDP* in leaves, and this gene's expression abundance showed different degrees of down-regulation under control, Q8 and Q8+NaHS treatments (Fig. 9H). The *GmFBP* expression level of 13.3 fold difference was detected by Q8+NaHS treatments compared with control treatment under SW condition. In control and Q8+NaHS treatments, the expression level of *GmFBP* was increased by the decrease of water content, but NaHS and Q8 treatments exhibited a trend of increasing first and then decreasing (Fig. 9I). In order to further elucidate the mechanism of H_2S promoted nodulation in soybean, we examined the expression of several nodulation marker genes including *GmENOD40* , *GmERN* , *GmNIN1a* , *GmNIN2a* , and *GmNIN2b* in soybean roots. The qRT-PCR results demonstrated that H_2S stimulated the expression levels of these genes in soybean roots. For instance, under whatever water condition, Q8 and Q8+NaHS treatments greatly elevated the expression levels of*GmENOD40* , *GmERN* , *GmNIN1a* , *GmNIN2a* , and*GmNIN2b* (Fig. 10A-E) in the inoculated roots during the entire treatment period. As for control and NaHS treatments did not give rise to any notably difference in the non-inoculated soybean roots.

Multivariate analysis of metabolome in leaves

To further study the effect of H_2S and rhizobia on water deficiency, a metabolomic analysis was performed in the control, Q8 and Q8+NaHS -treated soybean seedlings under NW and SW conditions. The OPLS-DA results (Fig. 11A, C and E, Fig. S2A, B, Fig. 12A, B) showed different responses in levels of metabolites by leaves of the three treatments to water stress. The importance of metabolites in sample discrimination associated with difference treatments were easily visible in the volcano plot which combined univariate and multivariate analyses (-log (P) obtained in the two-way ANOVA was plotted against the loading (p_{corr}) along axis (Fig. 11B, D, F). As can be seen from the volcano plot, in the control, SW condition resulted in more obvious metabolic response than NW condition (Fig. 11B), while the differential metabolites were less changed under the Q8 treatment (Fig. 11D). In addition, Q8+NaHS treatment seemed to have more up-regulation metabolites under SW conditions than under NW condition (Fig. 11F). The metabolites in the leaves were changed depending on the Q8 and Q8+NaHS treatments under water deficiency (Table 1). Our results showed that fatty acyl metabolites including methyl furfuracrylate and 2-Octenedioic acid synthesis were appreciably regulated by control treatment under SW condition. In addition, under SW condition, fatty acyl metabolites and isoflavone metabolites (Biochanin A) were sharply influenced by Q8 treatment. Furthermore, Q8+NaHS treatment regulated more metabolites than control and Q8 treatments under water

deficiency. We focused on separate analysis of lipid metabolism changes between different components (Table S2), and based on P [?]0.05, Q8+NaHS treatment significantly increased up-regulation metabolites compared with control.

Furthermore, the results showed that the addition of rhizobia appreciably affected the changes of metabolites under NW condition (Fig. S2C, D, Table S4). In SW condition, control/Q8 treatment led to a more distinct metabolic response than treatments with Q8/Q8+NaHS (Table 2). We could visually observe the change of metabolites under different treatments by the volcano plot (Fig. 12C, D). Under SW condition, there were 37 different metabolites based on P [?]0.001 in the comparison between control and Q8 treatments (Table 2). Among them, we separately analyzed the composition of lipids among different treatments (Table S5). The number of up-regulated lipid metabolites were remarkably increased between control and Q8 treatments under SW condition. Under SW condition, the up-regulated lipid metabolites were similar to the NW condition between Q8 and Q8+NaHS treatments. It is worth noting that metabolomics that was indicated H_2S and rhizobia synergistically regulated lipid metabolites under SW condition, including PE, PG, agavoside A, and dephospho-CoA in leaves, showing an effective H_2S and rhizobia synergistically regulation and improved tolerance to water deficiency.

Discussion

H_2S and rhizobia jointly regulate the growth of plant, photosynthesis, and chlorophyll fluorescence under water deficiency

Previous studies showed that rhizobia could promote plant growth and increase biomass accumulation under metal contaminated environment (Shen et al., 2019). Rhizobia also improve alfalfa productivity and increase biomass in different alfalfa cultivars under salt condition (Bertrand et al., 2015). In the present study, water deficiency reduced the shoot and root biomass, but rhizobia can significantly alleviate the decrease of water deficiency-induced biomass in soybean seedlings (Fig. 1A). Moreover, our data indicated that 100 μM NaHS and the inoculation of rhizobia substantially increased the shoot and root biomass of soybean plants under water deficiency condition (Fig. 1B, C). similarly, the study of Zhang et al. (2020) showed that H_2S and rhizobia can jointly regulate the biomass and growth in soybean under N deficiency. These results suggested that H_2S and rhizobia jointly alleviated stressful environments in plants, likely by increasing biomass yield and maintaining a higher level of nitrogen fixation. Therefore, we concluded that the interaction of H_2S and rhizobia more effectively enhanced plant growth under water deficiency condition.

Plants could avoid leaf water loss by adjusting stomatal conductance and transpiration (Jin & Pei, 2015; Stanton & Mickelbart, 2014). In the present study, we found that the leaf RWC was increased by Q8+NaHS than that of the control plants under SW condition (Fig. 1E). In response to drought, higher leaf RWC in leaves may be due to reduce water loss in most plants (Yin et al., 2013), as exemplify by phenomenons showing high biomass under water deficiency in Q8 and Q8+NaHS treatments, which tended to have an increased leaf RWC when water availability decreased. Besides, the leaf RWC was increased by NaHS in *S. oleracea*seedlings under drought condition (Chen et al., 2016). These results clearly indicated that H_2S and rhizobia markedly alleviated water loss of plant leaves under water deficiency condition.

H_2S promoted chlorophyll synthesis and reduced chlorophyll loss under environmental stress (Zhang et al., 2010; Zhang et al., 2009). For instance, under aluminum stress the increase of chlorophyll content caused by exogenous H_2S substantially promoted the growth of rape (Qian et al., 2014). Moreover, exogenous H_2S remarkably mitigated the deterioration of chlorophyll content under cadmium stress (Tian et al., 2016). Similarly, Mostofa et al. (2015) reported the restoration of chlorophyll content with exogenous H_2S in rice under cadmium stress. Further, Ding et al.(2019) also claimed that the higher plant growth was due to the increase of chlorophyll content by H_2S-mediated under salinity stress. Our results also indicated that H_2S and rhizobia jointly mediated the increase in chlorophyll content improved the growth of soybean plants under water deficiency conditions, suggesting that H_2S and rhizobia enhance the synthesis complexes and protein molecules of chloroplasts and mitochondria. However, it is noteworthy that the chlorophyll content was increased gradually with increasing water deficiency (Fig. 1D). Similar results had reported

that drought stress could induce the increase in photosynthetic pigments in*Arabidopsis thaliana* (Gamar et al., 2019). However, many papers reported a decrease in chlorophyll content due to drought stress in other legumes (Basal et al., 2020; Buezo et al., 2019; Hao et al., 2013). Munawar et al. (2019) reported a notable decline in photosynthetic pigments in drought-stressed broccoli plants. Naz et al. (2016) found the decline in chlorophyll molecules in cucumber under drought. Drought stress significantly diminished chlorophyll molecules in radish (Akram et al. 2015). Some possible explanations for this discrepancy included the differences in plant species, the drought stress intensity and duration of treatment. In this study, under SW condition, the Pn, Tr, and Gs were higher in H_2S and rhizobia treatment than in the control treatment (Fig. 3A, B, D). These results showed that Q8+NaHS-treated plants exhibited a high photosynthetic rate compared with the control treatment under water deficiency condition. The major reason of low photosynthesis rate is caused by inadequate ribulose-1, 5-bisphosphate (RUBP) synthesis, as a result of decreased ATP synthesis (Lawlor, 2002). Hence, the higher photosynthesis by Q8+NaHS-treated plants under water deficiency condition could be related to the positive role of H_2S and rhizobia on cellular ATP production in soybean leaves. Moreover, H_2S acted as modulator of PSII activity in leaves. Specifically, ETR, PSII, and Fv/Fm parameters were increased by Q8+NaHS treatment (Fig. 4A, B, and D), indicating that the photochemical efficiency of PSII was increased by H_2S and rhizobia. These were consistent with the increase in photosynthetic rate by Q8+NaHS treatment under water deficiency. In general, above results suggested that the interaction of H_2S and rhizobia jointly enhanced photosynthesis and alleviated the inhibition of soybean biomass due to lack of water.

H_2S and rhizobia regulates nodule formation and nitrogenase activity in soybean plants under water deficiency

Symbiotic nitrogen fixation increases the available nitrogen content of plants and promotes legumes growth and development. Many small signaling molecules, such as NO, H_2O_2, and phytohormones have been reported to be involved in the regulation of nodulation between legumes and rhizobia (Hérouart et al., 2002; Puppo et al., 2013). In this experiment, our results showed water deficiency significantly inhibited the accumulation of nodules weight, endogenous H_2S content, and nitrogenase activities (Fig. 2C, D and E), suggesting that water deficiency affected the establishment of symbiotic system between rhizobia and soybean. Besides, under SW condition, the endogenous H_2S content was higher in the Q8+NaHS-treated plants than in the control treatment (Fig. 2D, E). Similarly, Chen et al. (2016) found that NaHS application enhanced the endogenous H_2S content in *S. oleracea* leaves under drought stress. There was no significant difference in the nodule nitrogen fixation area between NaHS+Q8 and the Q8 treatments under NW and MW conditions (Fig. S1). The nodule nitrogen fixation area was notably reduced under SW condition compared with NW condition. Interestingly, the nitrogen fixation area was partially rescued in NaHS-treated nodules under SW condition (Fig. S1). These results indicated that under water deficiency condition H_2S could alleviate water deficiency-induced the decrease of the nitrogenase activity. Additionally, an interesting phenomenon was found that under MW condition the nodules number was greatly increased, but nodules biomass was not obviously affected under the same condition (Fig. 2B, C). The possible reason was that water deficiency stimulated root nodulation to resist water stress. Additionally, previous studies have shown that plant can continue to nodule in the water deficiency by stimulating the early nodulation factor (Paula et al., 2016). In the present study, we investigated the expression of symbiosis-related genes in root nodules. *GmENOD40* is a downstream component of NFs perception (Ferguson et al., 2010), which is expressed in peripheral cells, cortical cells, nodule primordia and developing nodules of root vascular bundles (Ferguson & Mathesius, 2014). Charon et al. (1999) reported that the change of *ENOD40* expression abundance could affect nodulation, suggesting that it plays an important role in the organogenesis of nodules. In the present study, the expression abundance of *GmENOD40* was up-regulated by H_2S and rhizobia in soybean roots under whatever water condition (Fig. 10A). Besides, another nodulation marker genes involved in the pathway of NFs nodulation, such as *GmERN* , was also activated by H_2S and rhizobia (Fig. 10B). *NIN* is essential for nodule organogenesis and nitrogen-fixing symbiosis in *Medicago truncatula* roots (Tatiana et al., 2015). Under any water condition, we found that the expression levels of three *NIN* genes were up-regulated by Q8+NaHS treatment in soybean root (Fig. 10C, D, E). Above results showed that H_2S and rhizobia stimulated the expression of *GmENOD40* , *GmERN* , and *GmNIN* genes under water deficiency

condition in soybean roots. Moreover, the moderate water deficiency may stimulate the nodule formation in the soybean symbiosis system and help plants to adaption the water deficiency environment. Therefore, our results suggested under water deficiency condition H_2S promoted nodule development and enhanced water-deficiency tolerance in the soybean-rhizobia symbiotic system.

H_2S and rhizobia synergistically increase antioxidant defense capacity of soybean leaves under water deficiency

Plants are often subjected to continuous threat from toxic ROS and lipid peroxidation. In order to cope with environment stress, plants increase their resistance by activating their antioxidant defense system (Farooq et al., 2019; Niu et al., 2012). ROS-generated oxidative injuries in plants were reflected by the MDA content, a byproduct of lipid peroxidation (Sato et al., 2011). We found that the over accumulation of MDA was observed under SW condition. Compared with the control, the Q8+NaHS-treated plants did not maintain lower levels of MDA content to some extent under water deficiency (Fig. 5B). This is contrast with the study conducted by Wang et al. (2016), who found that there is notably lower MDA content in the leaves of alfalfa with rhizobia than control seedlings. These results suggested that severe water deficiency could damage the membrane structure and lead to excessive oxidative damage in soybean leaves. Furthermore, it was reported that H_2S reduced membrane peroxidation by regulating the activities of antioxidant enzymes in rice under low temperature stress (Mostofa et al., 2015), and also regulated the antioxidant system of pepper to relieve zinc toxicity (Kaya et al., 2018). In the present study, H_2S and rhizobia synergistically decrease in H_2O_2 levels through increasing activities of antioxidants in soybean leaves under water deficiency condition (Fig. 5C). Further proof was provided by the assay of antioxidant enzyme activities in soybean leaves tissues. Our results showed that the activities of CAT and POD were markedly increased in soybean treated with H_2S and rhizobia compared with the control treatment under water deficiency (Fig. 6B, C), indicating that plants maintained high levels of antioxidants activities to remove water stress induced-excess ROS. In other studies, it has been shown that the balance among POD, CAT and SOD is crucial for determining the steady-state level of H_2O_2 and superoxide radicals (Dong et al., 2019; Li et al., 2020). Together, these results suggested that the activities of antioxidants played a vital role in alleviating the oxidative damage of soybean under water deficiency. Although plants maintained high levels of CAT and POD activities to some extent by Q8+NaHS treatment under water deficiency, the activity of SOD did not markedly increase. Notably, this is little decrease for the OFR content to some extent for H_2S and rhizobia treatment under water deficiency. Meanwhile, compared with NW condition, the content of OFR was increased by H_2S and rhizobia under SW condition (Fig. 5 D). In addition, the transcript abundances of several antioxidant defense-related genes were increased by H_2S and rhizobia under SW condition (Fig. 9). Importantly, *GmSOD2* , which is encoded the ron-SOD2, exhibited higher expression levels in Q8+NaHS-treated plants than in the control plants under SW condition. This may be due to the fact that SOD, as an inducible enzyme, produces more superoxide anion and induces *SOD*gene expression under water deficiency condition. These results revealed that H_2S and rhizobia are more efficient in mitigating the damage caused by ROS in soybean under water deficiency.

The AsA-GSH cycle plays a vital role for plant to resist oxidative damage (Avashthi et al., 2018; Nanda & Agrawal, 2016). It is reported that *Brassica napus* plants can be protected from salt induced oxidative stress by regulating ASA-GSH pathway. (Hasanuzzaman et al., 2018). Furthermore, Chen et al. (2011) reported that H_2S may lead to the accumulation of GSH in plant tissues. Our results showed that water deficiency altered the redox status of AsA and GSH in soybean seedlings under water deficiency condition (Fig. 7). Moreover, the mRNA abundance of 1-Cys peroxiredoxin and glutaredoxin, which are encoded by*GmPrx* and *GmGrx* , are higher in the Q8+NaHS treatment plants than control plants under SW condition. This may corroborate why the H_2S and rhizobia show higher GR activity under water deficiency (Fig. 7I). Thus, the decline in the redox status levels by water deficiency is either due to the serious damage of plants. These results clearly revealed that water deficiency caused the alteration in the redox status of the cell by interfering with AsA and GSH pools in legumes. Furthermore, we found that H_2S and rhizobia synergistically enhanced the AsA-GSH cycle by regulating the activities of APX, GR, and DHAR in soybean under water deficiency (Fig. 6D, 7H, I). Similar results have been reported that the application of exogenous H_2S regulated the metabolism of AsA and GSH in wheat leaves by increasing the activities of APX, GR and DHAR under

water stress (Shan et al., 2011). Previous studies have shown that the higher accumulation of the AsA-GSH cycle and APX activity synergistically reduced the excess production of H_2O_2 in cashew plants (Lima et al., 2018). Khan et al. (2017) reported that NO-induced H_2S alleviated osmotic stress in wheat seedlings by enhancing the activities of APX and GR. Interestingly, the regeneration of GSH from oxidized glutathione (GSSG) is catalyzed by GR and improved the antioxidant capacity of cells (Hasanuzzaman et al., 2017). These results suggested that H_2S and rhizobia synergistically triggered the up-regulation of the AsA-GSH cycle related enzymes under water deficiency, which further enhanced abiotic stress tolerance in plants.

H_2S and rhizobia synergistically regulate osmoprotectants to adapt to water deficiency

Osmotic adaptation ability under water stress seems to be of particular importance to turgor pressure and plant growth (Yang et al., 2019). Soluble sugars and N-rich compounds such as GB and proline constitute part of the compounds accumulated in the cytoplasm of plant cells under drought conditions, which helped to maintain a low osmotic potential inside the cells (Ashraf & Foolad, 2007; Gomes et al., 2010; Shan et al., 2011). The increase in soluble saccharides concentration facilitates plants to resist environmental stress (Jha & Subramanian, 2018). In the present study, our results showed that the accumulation of sucrose, fructose, and glucose in the leaves of soybean treated with H_2S and rhizobia was higher than control treatment under SW condition (Fig. 8A-C), suggesting that H_2S and rhizobia enhanced water deficiency resistance by regulating the accumulation of soluble sugars, especially the content of fructose. Additionally, fructose-1, 6-bisphosphatase (FBP) is an important regulatory enzyme in the gluconeogenesis pathway. The FBP activity can regulate the gluconeogenesis pathway and is related to the amount of glucose released. Similarly, Rivero et al. (2014) and Chen et al. (2016) reported that salt and drought stress could specifically up-regulated the expression levels of the *FBP* gene in tomato and soybean. Our results showed the higher expression levels of *GmFBP* was found in in the Q8+NaHS treatment plants compared with those in the control treatment under SW condition, indicating FBPase played a very important for glucose regeneration, which is the main carbon skeleton in trehalose and starch (Rivero et al., 2014). Unlike the increase in sucrose content, the expression of *GmSUS* gene was down-regulated by Q8+NaHS treatment in soybean leaves under water deficiency condition (Fig. 9G). Therefore, we speculated that this phenomenon may be attributed to the enhancement of glucose and fructose as a result of increasing the hydrolysis of the sucrose.

GB and PRO are major organic osmolytes, which are involved in regulating the response to environmental stress in plants (Chen & Jiang, 2010; Zhang & Becker, 2015). GB mainly accumulates in the chloroplast and is involved in the maintenance of PSII efficiency under water stress conditions (Ben et al., 2008). We found that the GB content was significantly increased by Q8+NaHS in soybean leaves under the SW condition (Fig. 8E). A previous study showed that the GB accumulation in the chloroplast is more effective than that in other cellular compartments in protecting plants against oxidative stress (Park et al., 2010). In addition, exogenous GB treatment can prevent salt-induced excess ROS from damaging organelle structures, such as chloroplasts and mitochondria (Ashraf & Foolad, 2007). These results suggested that H_2S and rhizobia prevented excess ROS production through promoting the increase of GB during water deficiency, and GB may have a prominent role in osmotic regulation. It is widely recognized that the accumulation of PRO decreases cell osmotic potential resulting in compel plants to absorb water from the outside to maintain the stability of cell membranes and adjust (Verbruggen & Hermans, 2008). PRO accumulation was an adaptation of plants to adversity and plays an important role in improving plant resistance (Chen et al., 2016; Zhang & Becker, 2015). H_2S and rhizobia markably increased the accumulation of PRO in soybean plants exposed to SW condition (Fig. 8D), suggesting the proline accumulation may protect the stressed plant from dehydration and stabilize its subcellular structure under water deficiency condition. The H_2S and rhizobia-mediated PRO accumulation might have improved the osmotic adjustment restoring soybean plants' growth under water deficiency. In addition, maize seedlings pretreated with H_2S manifested a more significant increase in proline contents by increasing the activities of pyrroline-5-carboxylate synthase and substantially reducing proline dehydrogenase activities (Li et al., 2013). Exogenous H_2S induced a noteworthy increase in the foxtail millet's endogenous proline levels under Cd toxicity (Tian et al., 2016). Similarly, Kolupaev et al. (2019) reported an increase of proline accumulation in wheat with H_2S pretreatment under drought. These results showed that the interaction of H_2S and rhizobia could improve the water-deficiency tolerance by influencing the

accumulation of osmoprotective compounds in soybean.

H_2S responds to water deficiency by regulating lipid metabolism in leaves in a soybean-rhizobia symbiotic system

Non-targeted metabolomics data showed that soybean leaf metabolome had high plasticity as a strategy to regulate its metabolism under water deficiency condition. Previous studies indicated that metabolites appeared as biomarkers during the symbiosis of plants and microorganisms and have become an effective method of measuring plant performance (Fernandez et al., 2016). Moreover, the key metabolites (carbohydrates, amino acids, lipids, cofactors, nucleotides, peptides and secondary metabolites) were regulated and accumulated in plants in response to drought and high temperature stress (Das et al., 2017; Guy et al., 2007; Loskutov et al., 2017; Shulaev et al., 2010). In the present study, physiological data showed that H_2S and rhizobia could regulate membrane lipid peroxidation and protect plants from severe oxidative damage. Interestingly, there have been significant changes in the regulation of lipids and lipid metabolites in metabonomics data. In this experiment, the up-regulated lipids and lipid metabolites of Q8-treated plant were markably increased compared with control under SW condition, and compared with Q8+NaHS-NW the metabolic compounds of Q8+NaHS-SW also changed significantly (P [?]0.05) (Fig. 11). Under the SW condition, we found that the up-regulated metabolites including PA, PG, galactaric acid, jaceidin 4'-glucuronide, and methyl furfuracrylate were notably increased by the inoculation of rhizobia in leaves (Fig. 12, Table 2). Furthermore, H_2S and rhizobia synergistically regulated lipid metabolites including PE, PG, agavoside A, and dephospho-CoA in leaves under SW condition. These results may indicate that H_2S and rhizobia synergistically showed more metabolites to participate in the regulation of water deficiency. Previous study reported that the metabolism involved in osmotic adjustment (proline, etc.) and active oxygen removal (L-glutamine and γ-L-glutamyl L-glutamate) was appreciably increased in tenuiflora seedlings inoculated with arbuscular mycorrhiza under alkali stress, suggesting that mycorrhizal colonization enhanced the alkali tolerance of plants (Yang et al., 2020). As expected, rhizobia catalyzed more metabolites in response to water stress under water deficiency. Phospholipid PG exists on the thylakoid membrane and participates in the photosynthesis of plants. Our study was in line with view taken by Sun et al. (2010) opinion, who pointed out that the increase of PG slowed the damage caused by salt stress in tomato plant. In addition, Jiao et al. (2018) found that soybean roots could resist neutral salt stress by regulating the metabolism of amino acids, carbohydrates and polyols. Of course, we also found that under water deficiency condition the H_2S and rhizobia synergistically enhanced the metabolism of nutrients, including amino acids and organic acids (allantoic acid, D-pantethine, pentosidine) in soybean leaves. Nowadays, many lipids including phosphatidic acid, fatty acid, inositol phosphate, lysophospholipid, diacylglycerol, oxylipid, sphingolipid and N-acylethanolamine were found to play an important role in the signal transduction in plant response to abiotic stress (Chao et al., 2011; Kang et al., 2010; Kilaru et al., 2011; Wang, 2004; Zhang et al., 2019). Previous studies reported that lipid-mediated signal transduction responds to various environmental stresses (such as temperature, water shortage, salinity, etc), Phospholipase D and phosphatidic acid-mediated signal transduction results in less water loss by promoting the closure of stomata, thus becoming the key lipid in response to stress (Ji et al., 2018; Zhao, 2015). These results suggested that the H_2S and rhizobia synergistically regulated metabolism of nutrients in leaves, including lipid and organic acids, which improved the ROS detoxification capacity, membrane stability and water tolerance.

Conclusion

Based on the above results, we proposed a model in which H_2S and rhizobia cooperate with each other to positively affected water deficiency. As shown in Fig. 13, we proved that the interaction of H_2S and rhizobia have promoted the available nitrogen content of plants through symbiotic nitrogen fixation, which was used to reduce the damage caused by water deficiency. This interaction also responds to the accumulation of reactive oxygen species by regulating changes in plant metabolites, activating the oxidative defense system, increasing the expression level of related antioxidant genes, and regulating the expression of osmoprotectants related genes, thereby enhancing the photosynthesis of plants and improving the plant growth. In addition, the lipid metabolites in leaves may play an important role in activating the defense system to cope with

water deficiency, which together provided a better protective effect against oxidative damage for plants.

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Figure Captions

Figure 1 Effects of H_2S and rhizobia on plant phenotype (A) and biomass (B, C). The SPAD (D) and leaf RWC (E) of leaves by four different treatments changed under water deficiency. Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at$P < 0.05$.

Figure 2 Effect of H_2S on nodulation size (A), number of nodules (B), nodule biomass (C), endogenous hydrogen sulfide content (D), and acetylene reduction assay (E) of soybean under water deficiency. Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at $P < 0.05$.

Figure 3 Effects of H_2S and rhizobia on photosynthetic parameters of soybean plants under water deficiency. Net photosynthetic rate (Pn, A), stomatal conductance (Gs, B), intercellular CO_2 concentration (Ci, C), transpiration rate (Tr, D), stomatal limit value (Ls, E) and instantaneous moisture utilization rate (WUE, F). Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at$P < 0.05$.

Figure 4 Effects of H_2S and rhizobia on fluorescence parameters of soybean plants under water deficiency. Electronic transport ratio (ETR, A), quantum yield of PSII photochemistry (PSII, B), NPQ (C), the ratio of variable fluorescence to maximum fluorescence (Fv/Fm, D). Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at $P < 0.05$.

Figure 5 Effects of hydrogen sulfide and rhizobia on endogenous hydrogen sulfide (A), lipid membrane peroxidation (MDA, B), hydrogen peroxide (H_2O_2, C), and superoxide anion (OFR, D) contents in plant leaves under water deficiency. Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at $P < 0.05$.

Figure 6 Effects of hydrogen sulfide and rhizobia on superoxide dismutase (SOD, A) peroxidase (POD, B) catalase (CAT, C) ascorbate peroxidase (APX, D) in leaves of soybean plants under water deficiency. Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at$P < 0.05$.

Figure 7 The effect of H_2S and rhizobia inoculation on the GSH content (A), the GSSG content (B), the ratio of GSH/GSSG (C) , the AsA content (D), the DHA content (E) , the ratio of AsA/DHA(F), MDHAR activity (G), DHAR activity (H), and GR activity (I) in leaves of soybean plants under water

deficiency. Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at $P < 0.05$.

Figure 8 Effects of the addition of exogenous H_2S donor NaHS and rhizobia on the contents of Sucrose (A), Fructose (B), Glucose (C), Proline (PRO, D) and Glycine betaine (GB, E) in soybean leaves under water deficiency. Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at $P < 0.05$.

Figure 9 Effects of H_2S and rhizobia on the expression level of *GmCAT* (A), *Gm* SOD1 (B), *GmSOD2*(C), *GmPrx* (D), *GmGrx* (E), *GmBADH* (F), *GmSUS*(G), *GmUDP* (H), *GmFBP* (I) gene in soybean leaves under water deficiency. Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Data were expressed as the mean ± *SE* . And columns marked with different letters indicated significant differences at$P < 0.05$.

Figure 10 Gene expression level of symbiotic related genes. Relative expression levels of *GmENOD40* gene (A), *GmERN*gene (B), *GmNIN1a* gene (C), *GmNIN2a* gene (D), and*GmNIN2b* gene (E) are displayed in multiple line charts with symbols. Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%. Values are means ± *SE* (n = 9).

Figure 11 Response of non-targeted metabolomics to water deficiency in soybean leaves. (A) OPLS-DA is used to distinguish the difference between soybean leaves Control-SW/Control-NW samples. (B) Volcano plot (difference between -$\log_{10}P_{\text{ANOVA}}$ and OPLS-DA load) shows the best distinguishing metabolites in Control-SW/Control-NW related to the effectiveness of water deficiency. (C) Using OPLS-DA to distinguish the sample differences of Q8-SW/Q8-NW in soybean leaves. (D) Volcano plot (difference between -$\log_{10}$ $P_{\text{ANOVA}}$and OPLS load) shows the best distinguishing metabolites in Q8-SW/Q8-NW related to the effectiveness of water deficiency. (E) OPLS-DA is used to distinguish the sample difference of soybean leaves Q8+NaHS-SW/Q8+NaHS-NW. (F) Volcano plot (difference between -$\log_{10}$ P_{ANOVA} analysis of variance and OPLS load) shows the best distinguishing metabolites related to the effectiveness of water deficiency in Q8+NaHS-SW/Q8+NaHS-NW. The red horizontal dotted line indicates the threshold $P_{\text{ANOVA}} < 0.05$. The important variables that reach the threshold are marked in green (decreased when water is deficient) or red (increased when water is deficient). Control, without rhizobia or NaHS; NaHS, with 100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%.

Figure 12 Response of non-targeted metabolomics to soybean leaves under different treatments under water deficiency. (A) OPLS-DA is used to distinguish the difference of soybean leaf Control-SW/Q8-SW. (B) Volcano plot (difference between -$\log_{10}P_{\text{ANOVA}}$ analysis of variance and OPLS-DA load) shows the best distinguishing metabolites associated with water deficiency in Control-SW/Q8-SW. (C) Using OPLS-DA to distinguish the sample differences of soybean leaves Q8-SW/Q8+NaHS-SW. (D) Volcano plot (difference between -$\log_{10}$ P_{ANOVA}analysis of variance and OPLS load) shows the best distinguishing metabolites related to water deficiency in Q8-SW/Q8+NaHS-SW. The red horizontal dotted line represents the threshold$P_{\text{ANOVA}} <0.05$. Important variables that reach the threshold are marked in green (decrease when moisture is deficient) or red (increased when moisture is deficient). Control, without rhizobia or NaHS; NaHS, with

100 μM NaHS; Q8, with rhizobia inoculation; Q8+NaHS, with rhizobia inoculation and 100 μM NaHS. NW, normal moisture content 80%-90%; MW, moderate drought moisture content 50%-60%; SW, severe drought moisture content 20%-30%.

Figure 13 Schematic diagram of the mechanisms of drought tolerance response of soybean-rhizobia symbiotic system under the regulation of hydrogen sulfide under water deficiency.

Table 1 Significantly changed metabolites between different treatments under water deficiency in soybean leaves, P [?] 0.001.

Table 2 Changes of metabolites between different treatments under SW condition in soybean leaves, P [?] 0.001.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Xueyuan Lin and Juan Chen: designed the project. Xueyuan Lin: performed the experiments, analyzed the data, and wrote the manuscript. NiNa Zhang, YaMei Zhang, YiWen Zhao, WuYu Liu, WeiQin Zhang: provided suggestions on charts and figures. JianHua Zhang, GeHong Wei, and Juan Chen help to revise the manuscript. All authors discussed the results and helpd edit the manuscript.

Table 1 Significantly changed metabolites between different treatments under water deficiency in soybean leaves, P [?] 0.001.

	Metabolite	**Class**
Control-SW/NW	Serotinose	Organooxygen compounds
	Methyl furfuracrylate	Fatty Acyls
	2-Octenedioic acid	Fatty Acyls
Q8-SW/NW	Sarmentosin	Fatty Acyls
	Biochanin A	Isoflavonoids
Q8+NaHS-SW/NW	12-Hydroxydodecanoic acid	Hydroxy acids and derivati
	Nepetaside	Prenol lipids
	7-Hydroxyterpineol 8-glucoside	Organooxygen compounds
	20, 22-Dihydrodigoxigenin	Steroids and steroid deriva
	7-Methylguanosine 5'-phosphate	Purine nucleotides
	5-Hydroxy-4-methoxy-5-(1-oxo-9,12,15-hexadecatrienyl)-2(5H)-furanone	Carbonyl compounds
	LTB3	-
	7-Hydroxybutylidenephthalide 7-(6-malonylglucoside)	Organooxygen compounds
	PG(22:6(4Z,7Z,10Z,13Z,16Z,19Z)/20:2(11Z,14Z))	-
	VPGPR Enterostatin	Carboxylic acids and deriv
	5,7-Megastigmadien-9-ol glucoside	Fatty Acyls
	19-Hydroxy-PGE2	Fatty Acyls
	2,3-Dihydro-4-methylfuran	Dihydrofurans
	20-Hydroxy-PGF2a	Fatty Acyls
	PG(18:3(6Z,9Z,12Z)/16:1(9Z))	Glycerophospholipids

Table 2 Changes of metabolites between different treatments under SW condition in soybean leaves, P [?] 0.001.

	Metabolite	**Metabolite**
Control-SW/Q8-SW	1-Cyclopropyl-4-methyl-1,3-cyclohexanediol	1-Cyclopropyl-4-methy
	3-Oxodecanoic acid	3-Oxodecanoic acid
	2,3-Dihydro-4-methylfuran	2,3-Dihydro-4-methylf
	Tanacetol A	Tanacetol A
	(1(10)E,4a,5E)-1(10),5-Germacradiene-12-acetoxy-4,11-diol	(1(10)E,4a,5E)-1(10),5
	(±)-1,4-Nonanediol diacetate	(±)-1,4-Nonanediol dia
	Geniposidic acid	Geniposidic acid
	2,3-dinor Prostaglandin E1	2,3-dinor Prostaglandi
	Propofol glucuronide	Propofol glucuronide
	PG(18:3(6Z,9Z,12Z)/16:1(9Z))	PG(18:3(6Z,9Z,12Z)/1
	Oxoglutaric acid	Oxoglutaric acid
	Tipredane	Tipredane
	Maysin 3'-methyl ether	Maysin 3'-methyl ethe
	xi-2,3-Dihydro-2-oxo-1H-indole-3-acetic acid	xi-2,3-Dihydro-2-oxo-1
	Alpha-dihydroartemisinin	Alpha-dihydroartemisi
	(±)Abscisic Acid	(±)Abscisic Acid
	Muricin E	Muricin E
	Quinestrol	Quinestrol
	Soyacerebroside I	Soyacerebroside I
	(2-Methoxyethoxy)propanoic acid	(2-Methoxyethoxy)pro
	1,2,10-Trihydroxydihydro-trans-linalyl oxide 7-O-beta-D-glucopyranoside	1,2,10-Trihydroxydihyd
	Corchorifatty acid F	Corchorifatty acid F
	Eremopetasinorol	Eremopetasinorol
	5-Hexyltetrahydro-2-oxo-3-furancarboxylic acid	5-Hexyltetrahydro-2-o
	3-carboxy-4-methyl-5-pentyl-2-furanpropanoic acid	3-carboxy-4-methyl-5-p
	Corchorifatty acid D	Corchorifatty acid D
	Hypoletin 8-gentiobioside	Hypoletin 8-gentiobios
	PE(18:1(11Z)/18:3(6Z,9Z,12Z))	PE(18:1(11Z)/18:3(6Z
	Cinncassiol C	Cinncassiol C
	Cyclopassifloside VII	Cyclopassifloside VII
	6-Deoxocastasterone	6-Deoxocastasterone
	Zeranol	Zeranol
	10-Hydroxy-8-nor-2-fenchanone glucoside	10-Hydroxy-8-nor-2-fe
	Eriodictyol 7-(6-trans-p-coumaroylglucoside)	Eriodictyol 7-(6-trans-
	19-Hydroxycinnzeylanol 19-glucoside	19-Hydroxycinnzeylan
	Methyl furfuracrylate	Methyl furfuracrylate
	(R)-2-Hydroxycaprylic acid	(R)-2-Hydroxycaprylic
Q8-SW/Q8+NaHS-SW	Q8-SW/Q8+NaHS-SW	Tragopogonsaponin M
		PG(22:6(4Z,7Z,10Z,13