J Plant Growth Regul (2018) 37:5–15
DOI 10.1007/s00344-017-9704-8

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Exogenous Hydrogen Sulfide Ameliorates Seed Germination and Seedling Growth of Cauliflower Under Lead Stress and Its Antioxidant Role

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Received: 17 September 2016 / Accepted: 20 March 2017 / Published online: 12 April 2017
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Abstract Lead (Pb) is a widespread ecosystem pollutant and affects food security and public health. Hydrogen sulfide (H_2S) plays prominent roles in mediating a variety of responses to stresses. The effects of sodium hydrosulfide (NaHS), a fast releaser of H_2S, on cauliflower (*Brassica oleracea* L. var *botrytis* L. cv. Xiahua 60 d) seed germination and seedling growth under lead acetate stress were investigated in the present study. Pb (0.25 and 0.5 mM) stresses markedly inhibited seed germination and seedling growth, whereas the inhibition was effectively mitigated by NaHS application. Germination percentage, root length, shoot length, and fresh weight of single seedling significantly increased. In addition, NaHS elevated endogenous H_2S contents and reduced malonyldialdehyde, superoxide anion ($O_2\cdot^-$), and hydrogen peroxide (H_2O_2) production, thereby preventing oxidative damage from Pb or Pb and antioxidant enzyme inhibitor (diethyldithiocarbamate or 3-amino-1,2,4-triazole) dual stresses. The protective roles of NaHS were equivalent to the ROS scavengers, 4,5-dihydroxy-1,3-benzene disulfonic acid? and *N,N′*-Dimethylthiourea. Moreover, NaHS elevated non-protein thiols and total glutathione levels to chelate Pb or scavenge ROS directly. Our results demonstrated the strong protective and antioxidant roles of H_2S.

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Keywords Pb stress · H_2S · Cauliflower · ROS scavenging · Antioxidant effect

Introduction

Lead (Pb), a ubiquitous industrial and environmental pollutant, has been listed by the Agency for Toxic Substances and Disease Registry (ATSDR 2005) as a major heavy metal pollutant (Sidhu and others 2016a). It is easily absorbed by crops through contaminated air, water, and soil, and then threatens food safety and public health, particularly children and infants (Dewanjee and others 2015; Kim and others 2016; Wang and others 2015). Lead acetate (PbAc) is known to induce kidney damage, hepatotoxicity, neurotoxicity, oxidative imbalance, fibrosis, apoptosis, and reproductive system diseases (Abdel Moneim 2016; Abdou and Hassan 2014; Mahmoud and Sayed 2016; Rao and others 2016). In plants, Pb exposure also exerts deleterious effects on a series of physiological processes including seed germination, root elongation, cell division, photosynthesis, respiration, nutrients uptake and hormonal equilibrium, therefore affecting morphology, growth, and yield (Kanwal and others 2014; Lamhamdi and others 2011, 2013; Sharma and Dubey 2005; Wierzbicka and Obidzińska 1998; Yang and others 2016). Ultrastructural studies have identified that stomatal size and conductance were reduced, thylakoid membranes dissolved, starch grains vanished, cells shrunk, mitochondria and endoplasmic reticulum (ER) were destroyed, the structure of the nucleolus changed, and membrane permeability was modified in plant leaf and root cells under Pb toxicity (Ali and others 2014; Jiang and others 2014; Khan and others 2016b). High concentrations of Pb alter various gene expressions in different tissues (Kovalchuk and others 2005; Li and others 2015; Liu and

others 2009; Reis and others2015). Further, excess Pb in plant cells induces molecular damage mainly through triggering bursts of reactive oxygen species (ROS) including superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) (Hu and others 2015). These excessive ROS lead to irreversible peroxidation of lipids, proteins, photosynthetic pigments, RNA, and DNA, thereby affecting cell structure and viability (Verma and Dubey 2003). Plants have developed efficient antioxidant systems, including enzymatic and non-enzymatic antioxidants, to combat the toxicity of ROS and oxidative stress (Mittler 2002). Superoxide dismutase (SOD), as the first defense line, converts $O_2\cdot^-$ into H_2O_2, and then H_2O_2 can be quickly decomposed into O_2 and H_2O by catalase (CAT), peroxidase (POD), and ascorbate peroxidase (APX). Another H_2O_2 detoxification way is the Halliwell–Asada cycle with the participation of the antioxidants—ascorbate (AsA), glutathione (GSH), and glutathione reductase (GR) (Chen and others 2013b). Sustainable agriculture has emerged as a chief goal in the face of changing environmental conditions. So efficient approaches for detoxification of Pb are greatly needed. Some low cost and viable exogenous substances, such as salicylic acid (SA), abscisic acid (ABA), nitric oxide (NO), flavonoid preparation, ethylene diamine tetraacetic acid (EDTA), GSH, 20-hydroxyecdysone, and organic chelants have been used to assist in the detoxification for Pb stress (Chen and others 2007; Kanwal and others 2014; Kaur and others 2015; Khan and others 2016a, b; López-Orenes and others 2014; Lamhamdi and others 2016; Zhao and others 2009).

Just like NO, the new gaseous signal molecule hydrogen sulfide (H_2S) has been recommended for mediating a variety of physiological processes and responses to abiotic stresses (Chen and others 2011a; Fang and others 2014; Christou and others 2013; Hu and others 2012; Li and others 2013; Shi and others 2013; Wang and others 2012; Zhang and others 2011). H_2S effectively alleviated metal toxicity from copper (Cu), aluminum (Al), cadmium (Cd), and zinc (Zn) (Chen and others 2013a; Liu and others 2016; Mostofa and others 2015; Zhang and others 2008). The protective roles of H_2S against Pb stress were attributed to the elevated activities of SOD, POD, CAT, and APX and the decreased influx and transport of Pb in cotton (Bharwana and others 2014). So the decreases in electric conductivity (EC), malonyldialdehyde (MDA), and H_2O_2 contents demonstrated lowered oxidative damage by H_2S supplementation. Exogenous H_2S also had promotive effects on plant growth, root morphology, chlorophyll contents, net photosynthesis rate, and ultrastructural changes, such as integrated chloroplasts with well-developed thylakoid membranes and starch grains in mesophyll cells, and mature mitochondria, golgi bodies, and long ER in the root tip cells of *Brassica napus* (Ali and others 2014). Moreover, H_2S alleviated the hazardous impact of elements on plants by increasing cell wall-related pectin methylesterase (PME), expansions, citrate, or plasma membrane (PM) H^+-ATPase (Chen and others 2013a; Wang and others 2010). On the other hand, H_2S might serve as an prominent signal transmitter to activate downstream signal transductions and therefore attenuate the stresses from heavy metals (Li and others 2012a; Shi and others 2014). However, systematically understanding the protective mechanism of H_2S is quite complicated and challenging.

Cauliflower, belonging to Brassicaceae, is a popular vegetable grown throughout the world for its abundant proteins, minerals, vitamins, and metabolites which protect mankind from heart diseases and certain cancers (Keck and Finley 2004). Plant species related to Brassicaceae are generally considered as sensitive indicators or phytoremediators for their fast growth, higher biomass, and adsorptive ability of heavy metals (Ali and others 2014; Gupta and Gupta 2015; Sidhu and others 2016). However, the role of H_2S in alleviating Pb stress is still unknown at least when it comes to cauliflower. In this study, sodium hydrosulfide (NaHS), a common donor of H_2S, was applied to study the effects of exogenous H_2S treatments on seed germination and seedling growth of cauliflower under Pb stresses and the results would help understanding of the antioxidant effects of H_2S.

Materials and Methods

Plant Culture and Treatment

Seeds of cauliflower (*Brassica oleracea* L. var *botrytis* L.) cv. Xiahua 60 days were surface sterilized in 15% (v/v) sodium hypochlorite (NaClO) solution for 10 min followed by thorough rinsing. After being soaked for 3 h, every 20 seeds were cultivated on sterilized solid medium (1% agar) with 0, 0.1, 0.2, 0.3, 0.6, 0.9, 1.5, 3.0 mM NaHS in transparent glass bottles in duplicate for 12 h. Then the seeds were divided into three groups and treated by 0, 0.25, or 0.5 mM lead acetate [$Pb(CH_3COO)_2\cdot 3H_2O$/PbAc] on fresh media individually. Seeds were placed in the dark at (25 ± 1) °C for 3 days and then cultured under cycles of 14 h light/10 h dark photoperiod with 80–100 μmol/(m^2·s) illumination intensity for another 4 days. The phenotype was observed and germination percentage (GP) and biomass were measured.

For the physiological mechanism, cauliflower seedlings were cultured in half-strength Hoagland's solution for 20 days (Hoagland and Arnon 1950). The culture solutions were refreshed every 4 days. After that, the uniform seedlings were treated with 0 or 0.5 mM PbAc for 24 h, then transferred to the culture solutions added with NaHS, DDC

(diethyldithiocarbamate, a Cu/Zn-SOD inhibitor), Tiron (4,5-dihydroxy-1,3-benzene disulfonic acid, a $O_2\cdot^-$ scavenger), AT (3-amino-1,2,4-triazole, a catalase inhibitor), or DMTU (*N,N′*-dimethylthiourea, a H_2O_2 scavenger) for another 48 h to prevent direct precipitation of Pb. There were ten treatment combinations for enzymatic antioxidant assays: (1) CK, control without any reagents for treatment; (2) 0.2 mM NaHS; (3) 0.5 mM PbAc; (4) 0.5 mM PbAc + 0.2 mM NaHS; (5) 0.5 mM PbAc + 0.3 mM DDC; (6) 0.5 mM PbAc + 0.3 mM DDC + 0.2 mM NaHS; (7) 0.5 mM PbAc + 1.0 mM Trion; (8) 0.5 mM PbAc + 2 µM AT; (9) 0.5 mM PbAc + 2 µM AT + 0.2 mM NaHS; (10) 0.5 mM PbAc + 1 mM DMTU. Another 6 treatment combinations were designed for non-enzymatic antioxidant assays: (1) CK; (2) 0.2 mM NaHS; (3) 0.3 mM NaHS; (4) 0.5 mM PbAc; (5) 0.5 mM PbAc + 0.2 mM NaHS; (6) 0.5 mM PbAc + 0.3 mM NaHS. Leaves were used for physiological and biochemical parameter measurements.

Quantification of Leaf H_2S

H_2S contents in leaves were detected according to the previously described procedure (Christou and others 2013). Leaf samples (0.3 g) were grounded into powder with liquid nitrogen and homogenized in 1 ml potassium phosphate buffer (100 mM, pH 7.0) with 10 mM EDTA. After centrifugation at 12,000×*g* for 15 min at 4 °C, supernatants were collected. The initial absorbance at 412 nm was recorded with Multiskan Spectrum (Thermo, USA). Then 200 µl extraction was mixed with 4 µl of 20 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and absorbance at 412 nm was measured every 5 min. The steady values of 10 min were chosen for quantification and H_2S concentration was calculated based on a standard curve of NaHS reagent.

MDA and ROS Determinations

The concentrations of $O_2\cdot^-$ and H_2O_2 in cauliflower leaf samples were measured according to the regular methods (Jana 1981; Mishra and Singhal 1991). MDA is a byproduct of lipid peroxidation and its level was determined following the method described by Wang and others (2009) with small modifications. Fresh leaves (0.5 g) were ground with 5 ml 10% (w/v) trichloroacetic acid (TCA). After centrifugation at 8000×*g* for 10 min, 2 ml of the supernatant was collected and mixed with 2 ml 0.6% TBA solution (dissolved in 10% TCA). Mixtures were kept in a boiling-water bath for 15 min, and then immediately cooled and centrifuged at 8000×*g* for 10 min. Then the absorbances of the final supernatants at 450, 532, and 600 nm were recorded. The MDA contents were calculated as follows: ($\mu M\ MDA) = 6.45(A_{532} - A_{600}) - 0.56A_{450}$, and the final contents were presented as nmol/g fresh weight (Fw).

Measurements of Antioxidant Enzyme Activities

Cauliflower leaves (0.5 g) were homogenized in 3 ml ice-cold 50 mM potassium phosphate (pH 7.8) buffer containing 1% polyvinyl pyrrolidone (PVP, w/v) for enzyme extractions. The homogenates were centrifuged at 8000×*g* and 4 °C for 20 min and the supernatants were collected. Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured by its ability to inhibit photochemical reduction of nitroblue tetrazolium chloride (NBT) at 560 nm (Beauchamp and Fridovich 1971). One unit of SOD was defined as the amount of enzyme causing 50% inhibition of NBT reduction under light for 6 min. Catalase activity (CAT, EC 1.11.1.6) was assayed according to the reported method (Aebi 1983). The decrease of absorbance at 240 nm was recorded and the final CAT activity was estimated using the extinction coefficient of 39.4 l/(mmol·cm). One unit of enzyme activity was defined as the reduction of 1 nM H_2O_2 per minute. Peroxidase (POD, EC 1.11.1.7) activity was determined following the procedure described by Rao and others (1997). The increase of absorbance at 470 nm was read continuously for 2 min and the extinction coefficient was 26.6 l/(mmol·cm). One unit of POD activity was defined as the formation of 1 nM tetraguaiacol per minute. The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to the previously published method (Nakano and Asada 1981). Three ml of the reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM Na_2-EDTA, 0.5 mM AsA, 0.1 mM H_2O_2, and 300 µl enzyme extract. The decrease of absorbance at 290 nm was recorded and the extinction coefficient was 2.8 l/(mmol·cm). One unit of APX activity was defined as the oxidation of 1 nM ascorbate per minute. Glutathione reductase (GR, EC 1.6.4.2) activity was assayed in terms of the method described by Schaedle and Bassham (1977). The reaction mixture (2.5 ml) contained 100 mM Tris–HCl buffer (pH 7.8), 2 mM EDTA, 50 µM NADPH, 0.5 mM glutathione disulfide (GSSG), and 100 µl enzyme extract. The extinction coefficient for GR activity calculation was 6.22 l/(mmol·cm) and one unit of GR activity was defined as the reduction of 1 nM NADPH at 340 nm per minute.

Quantification of Non-protein Thiols (NPT) and Glutathione (GSH)

Leaf samples (0.2 g) were ground in 2 ml 5% (w/v) sulfosalicylic acid solution containing 6.3 mM diethylenetriaminepentaacetic acid (DPTA) under an ice bath. The mixture was centrifuged at 4 °C under 10,000×*g* for 10 min, and then the supernatant was immediately collected. Fifty micro liters of the supernatant was mixed with 100 µl 0.5 M K_2HPO_4 solution. Two minutes later, the initial absorbance at 412 nm was recorded. Then 5 µl DTNB solution (6 mM DTNB

dissolved in 100 mM phosphate buffer, pH 7.6, with 5 mM EDTA) was supplemented. Ten minutes later, absorbance at 412 nm was measured again. The steadily increased absorbance was used for calculating NPT concentration with an extinction coefficient of 13.6 l/(mmol·cm) (Mendoza-Cozatl and others 2008). Total GSH was assayed using an ELISA kit (S0053) from Beyotime Institute of Biotechnology (China). The assays were performed according to the manufacturer's manual. The standard calibration curve was plotted with 0.5–15 μM GSSG (Chen and others 2012).

Statistical Analysis

Values were expressed as means ± standard deviations (SD) of at least triplicate independent experiments and measurements. Significant differences analyses were performed with Student *t* test for pairs or one-way analysis of variance for groups (Duncan's new multiple range test).

Results

Exogenous H_2S Promoted Seed Germination and Seedling Growth of Cauliflower Under Pb Stresses

Under normal conditions, the germination percentage (GP), root length (RL), shoot length (SL), and fresh weight of a single seedling (SW) of 7-day-old seedlings of cauliflower 'Xiahua 60 d' were 90.0%, 6.21, 2.06 cm, and 0.0737 g, respectively (Fig. 1a–d). NaHS (0.1–0.9 mM, an H_2S fast releaser) pretreatment had slight effects on seedling growth (Fig. 1), whereas 1.5 and 3.0 mM NaHS significantly inhibited root elongation ($P = 0.0014$ or 0.0005, <0.01),

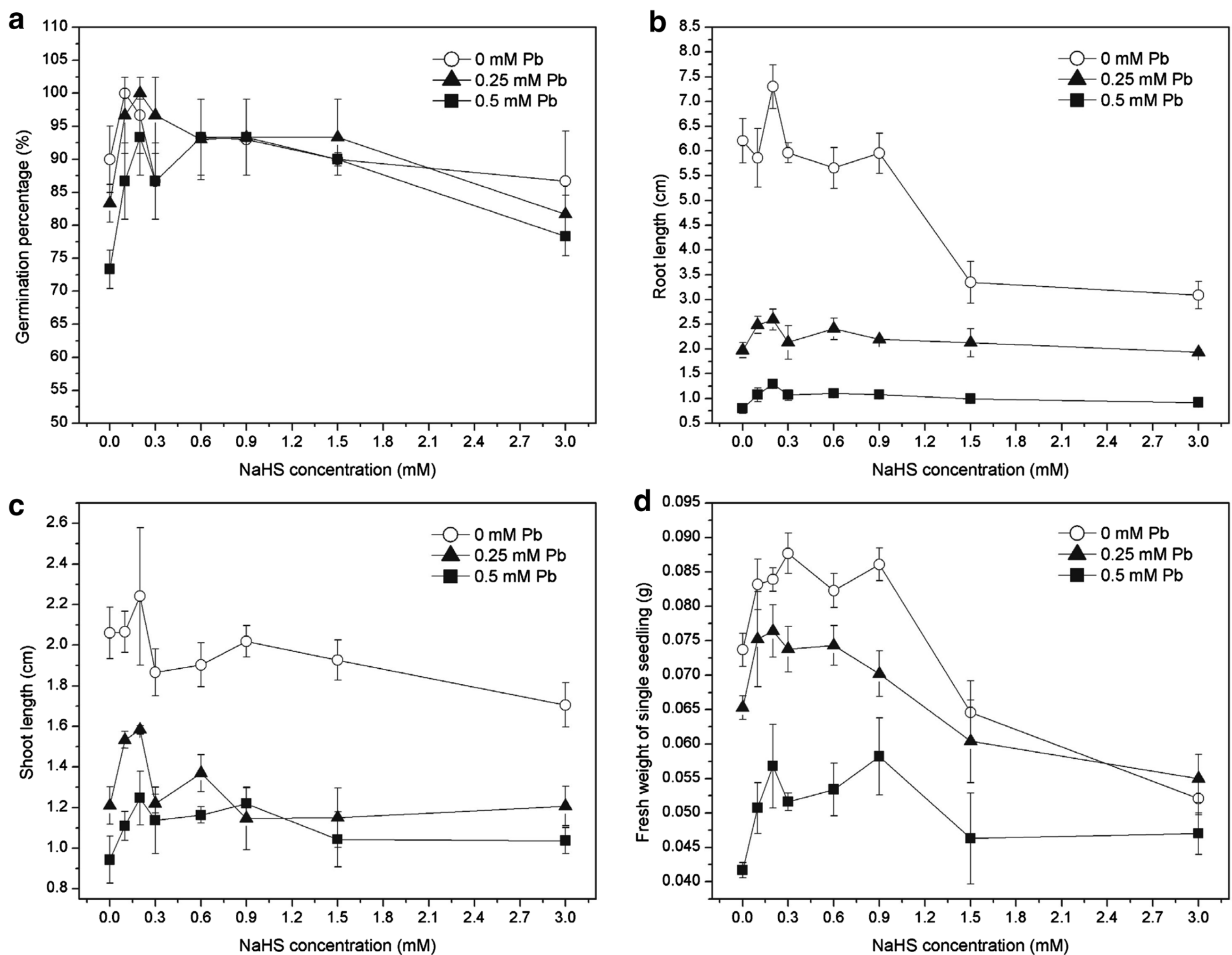

Fig. 1 Effects of exogenous H_2S on seed germination and seedling growth of cauliflower under Pb stresses. **a** GP; **b** RL; **c** SL; **d** SW. Seeds were pretreated with 0–3.0 mM NaHS for 12 h, then treated with 0, 0.25, or 0.5 mM PbAc for 7 days. Data are presented as the mean ± SD ($n = 20 \times 3$)

compared to the control level (Fig. 1b). Three mM NaHS also reduced SL and SW (Fig. 1c, d), indicating that higher concentrations of NaHS might be toxic to cauliflower seeds.

Pb (0.25 and 0.5 mM) significantly inhibited cauliflower seedling growth after seed germination (Fig. 1). RL was only 1.97 or 0.08 cm, with sharp decreases of 68.28 or 87.12% in comparison with the control. Meanwhile, GP, SL, and SW declined by 7.41 or 18.56%, 37.38 or 54.37%, and 11.80 or 43.01%, respectively. NaHS pretreatment at concentrations of 0.1–0.9 mM effectively promoted seed germination and seedling growth under Pb stresses whereas higher concentrations of 1.5–3.0 mM had no significant effect. NaHS (0.2 mM) showed the optimum effect, and GP, RL, SL, and SW increased by 20.00 or 27. 27%, 31.42 or 62.09%, 30.85 or 32.16%, 17.00 or 36.21%, respectively, compared to the condition of 0.25 or 0.5 mM Pb alone.

Leaf H_2S Contents

Under normal conditions, H_2S in leaves was kept at a low level of about 6.66 nmol/g Fw (Fig. 2). Exogenous root application of NaHS led to a significant burst of H_2S in leaves, up to approximately 30 nmol/g Fw. Pb exposure also induced some H_2S production and the concent of H_2S was as much as 2.11-fold of the control, but much lower than NaHS treatment alone. The $O_2\cdot^-$ scavenger, Tiron, and antioxidant enzyme inhibitors, DDC and AT, had no obvious effects on the changes of H_2S content in seedlings after Pb stress. The H_2O_2 scavenger, DMTU, treated after Pb stress, significantly enhanced H_2S level, compared to Pb treatment alone. When exogenous NaHS was supplemented to the solutions of Pb or Pb and antioxidant enzyme inhibitor co-treatments, H_2S contents in leaves were all greater than 30 nmol/g Fw (Fig. 2), proving the effective release of H_2S.

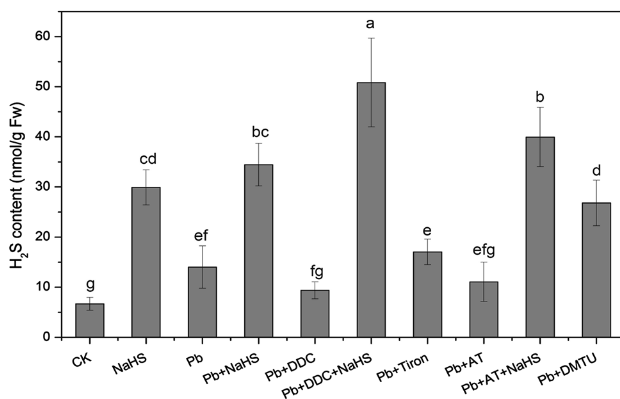

Fig. 2 Effects of 0.2 mM NaHS root treatment on leaf H_2S contents. (1) CK, control; (2) NaHS, 0.2 mM NaHS treated for 48 h; (3) Pb, 0.5 mM PbAc treated for 24 h; (4) Pb + NaHS, 0.5 mM PbAc treated for 24 h then transfered to 0.2 mM NaHS treatment for 48 h; (5) Pb + DDC, 0.5 mM PbAc treated for 24 h then transfered to 0.3 mM DDC treatment for 48 h; (6) Pb + DDC + NaHS, 0.5 mM PbAc treated for 24 h then transfered to 0.3 mM DDC and 0.2 mM NaHS treatment for 48 h; (7) Pb + Tiron, 0.5 mM PbAc treated for 24 h then transfered to 1.0 mM Tiron treatment for 48 h; (8) Pb + AT, 0.5 mM PbAc treated for 24 h then transfered to 2 μM AT treatment for 48 h; (9) Pb + AT + NaHS, 0.5 mM PbAc treated for 24 h then transfered to 2 μM AT and 0.2 mM NaHS treatment for 48 h; (10) Pb + DMTU, 0.5 mM PbAc treated for 24 h then transfered to 1 mM DMTU treatment for 48 h, the same as follows. Data are presented as the mean ± SD (n = 4). Values with *different lowercase* mean the significant difference at $P < 0.05$ level

Exogenous H_2S Alleviated Oxidative Stress Caused by Pb Stress

Exposure to 0.5 mM Pb caused severe oxidative stress to cauliflower seedlings. MDA, $O_2\cdot^-$, and H_2O_2 contents in leaves under Pb stress rose up to 2.04-, 2.39-, and 2.55-fold of controls (Figs. 3, 4). NaHS alone did not induce excessive MDA and $O_2\cdot^-$ production but slightly increased H_2O_2 content. However, the generation of ROS and MDA triggered by Pb was markedly diminished by NaHS treatment (Figs. 3, 4). The low oxidative damage proved the protective effect of H_2S.

For further investigations, antioxidant enzyme inhibitors and ROS scavengers were used in this study. When SOD activity was inhibited by DDC, $O_2\cdot^-$ could not be effectively converted into H_2O_2 and its production rate remained at a high level (Figs. 4, 5a). When NaHS was supplemented to this condition of Pb + DDC, the $O_2\cdot^-$ production rate decreased sharply to the level when $O_2\cdot^-$ was eliminated by its scavenger, Tiron (Fig. 4). In addition, when CAT activity was inhibited by AT, H_2O_2 decomposition was partly

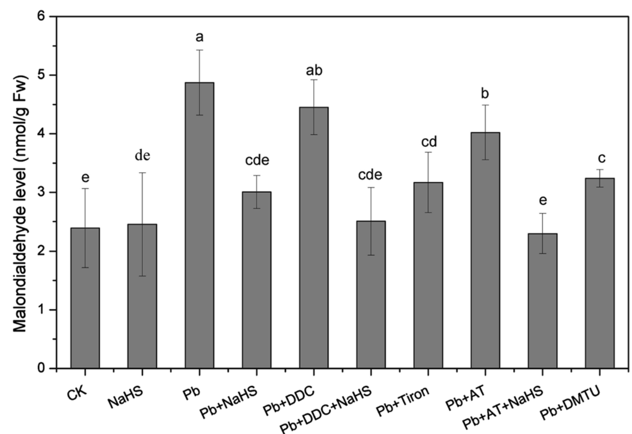

Fig. 3 Effects of H_2S, antioxidant enzyme inhibitors, and ROS scavengers on malondialdehyde (MDA) contents in the leaves of cauliflower seedlings under Pb stress. Data are presented as the mean ± SD (n = 4). Values with *different lowercase* mean the significant difference at $P < 0.05$ level

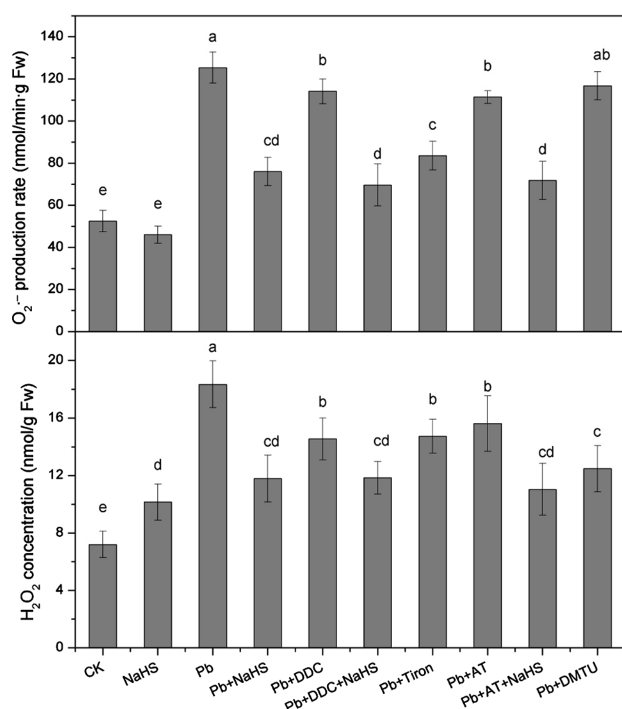

Fig. 4 Effects of H_2S, antioxidant enzyme inhibitors, and ROS scavengers on the superoxide anion ($O_2{\cdot}^-$) and hydrogen peroxide (H_2O_2) levels in the leaves of cauliflower seedlings under Pb stress. Data are presented as the mean ± SD ($n = 4$). Values with different lowercase mean the significant difference at $P < 0.05$ level

blocked. NaHS supplement reduced the H_2O_2 content and its role might be equivalent to DMTU, the H_2O_2 scavenger (Figs. 4, 5b). So we speculated that H_2S might quench ROS directly or indirectly to alleviate oxidative damage.

Effect of Exogenous H_2S on Antioxidant Enzyme Activities

Under Pb stress, cauliflower launched an antioxidant enzyme system to eliminate ROS. SOD, CAT, POD, APX, and GR were all notably activated (Figs. 3, 4). For control and 0.2 mM NaHS, the SOD activities were 137.48 and 132.43 units/g Fw. However, it significantly increased to 164.80 units/g Fw with the addition of Pb stress. However, NaHS did not strengthen SOD activity under Pb or Pb and oxidative dual stresses. The SOD activity recovered to 142.65 or 126.89 units/g Fw under conditions of Pb + NaHS or Pb + DDC + NaHS (Fig. 5a). There was a similar trend for CAT activity when it was inhibited by AT (Fig. 5b). Moreover, POD, APX, and GR activities all decreased with the presence of NaHS (Fig. 5c–e). All these data suggested that 0.5 mM Pb exerts severe oxidative damage on cauliflower seedlings whereas endogenous H_2S eliminates ROS and keeps them at low levels, thereby exhibiting strong protective effects. Nevertheless, it should be noted that the antioxidant effect of H_2S is not due to antioxidant enzymes.

Exogenous H_2S Elevated Non-protein Thiols and Glutathione Levels

In the absence of Pb stress, non-protein thiols (NPT) and total GSH concentrations in leaves remained at low levels (Table 1). With Pb exposure, synthesis and conversion of NPT and GSH elevated dramatically and increased even more with NaHS supplementation (Table 1), indicating the possible conversion of H_2S to GSH. So the elevated non-protein thiol-containing compounds assisted detoxification by chelating Pb or scavenging ROS and eventually ameliorated germination and growth.

Discussion

Pb is a widespread ecosystem pollutant and a highly toxic nerve poison (Kim and others 2016; Mahmoud and Sayed 2016). Pb causes a number of inhibitory and toxic effects on seed germination and seedling growth of plants (Ali and others 2014; Hu and others 2015; Lamhamdi and others 2011; Yang and others 2016). In the present study, significant decreases in all germination parameters of cauliflower seeds were observed under 0.25 and 0.5 mM Pb stresses, whereas the inhibition was mitigated by the applications of NaHS, an H_2S fast releaser (Fig. 1). NaHS elevated H_2S contents in leaves (Fig. 2) and reduced MDA, $O_2{\cdot}^-$, and H_2O_2 productions, thereby preventing oxidative damage from Pb or Pb and antioxidant enzyme inhibitor (DDC or AT) dual stresses (Figs. 3, 4). However, NaHS applications did not strengthen SOD, CAT, POD, APX, and GR activities but reduced them (Fig. 5a–e). So it is noteworthy that H_2S eliminated ROS not via antioxidant enzymes. In addition, the levels of MDA and ROS showed that the protective roles of H_2S were parallel to the ROS scavengers, Tiron, and DMTU (Figs. 3, 4). Moreover, NaHS elevated the levels of NPT and total GSH (Table 1). These results demonstrated the strong antioxidant role of H_2S.

NaHS is a commonly used donor of H_2S (Hancock and Whiteman 2016; Xie and others 2016). It enhances the endogenous H_2S content to high levels at 12–48 h (Li and others 2014a). Our results confirmed the effectiveness of NaHS on H_2S supply (Fig. 2). Chen and others (2011) analyzed the generation of H_2S from various sulfur- or sodium-containing compounds, including NaHS, Na_2S, Na_2SO_3, Na_2SO_4, $NaHSO_3$, $NaHSO_4$, and NaAC, and found that only NaHS could generate H_2S largely and steadily. In addition, 10 and 100 μM NaHS promoted *Spinacia oleracea* seedling growth, whereas 500 and 1000 μM NaHS showed inhibitive effects (Chen and others 2011). We also found

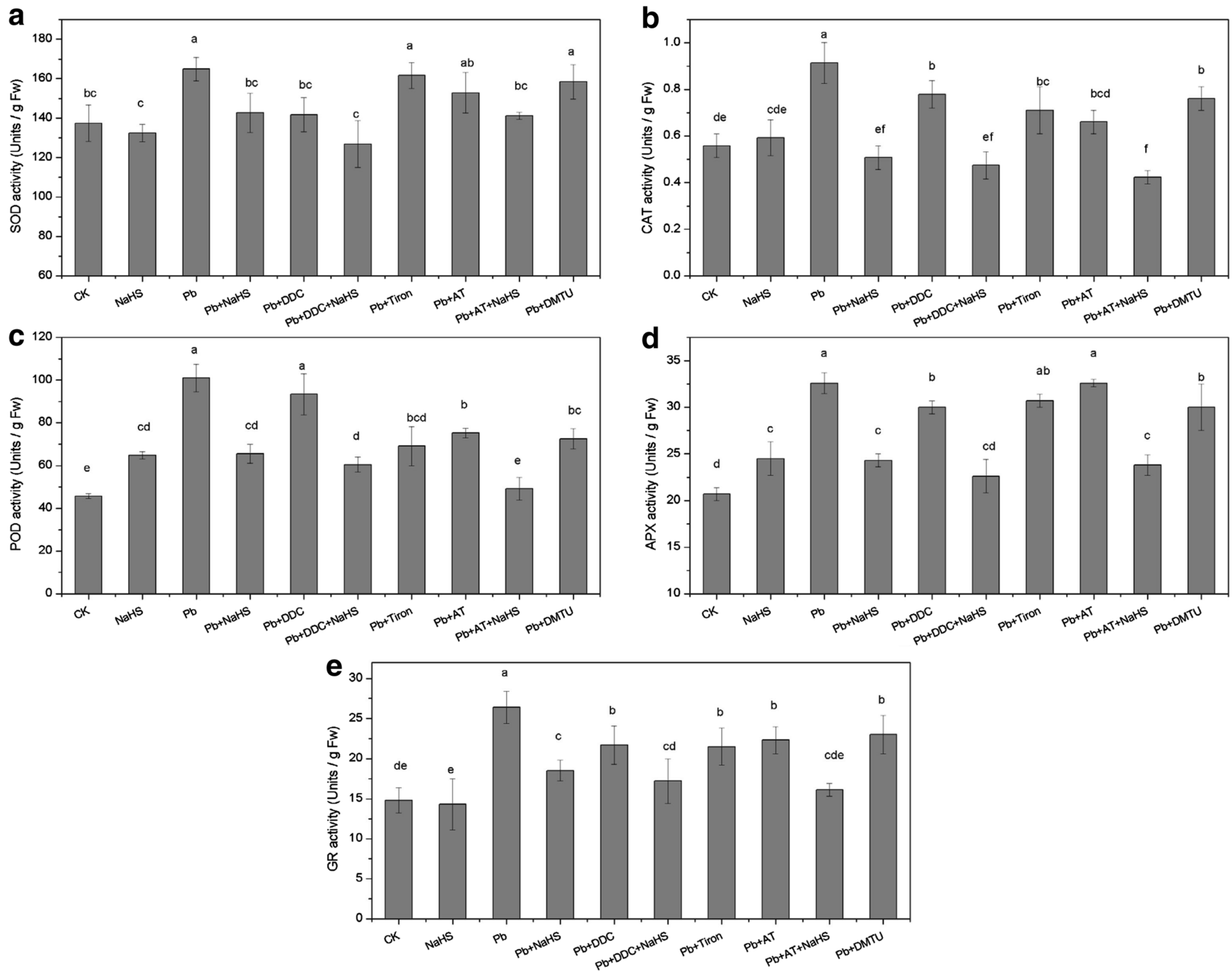

Fig. 5 Activity analysis of antioxidant enzymes in the leaves of cauliflower seedlings under Pb stress, antioxidant enzyme inhibitors, and ROS scavenger treatments. **a** SOD; **b** CAT; **c** POD; **d** APX; **e** GR. Data are presented as the mean ± SD ($n=4$). Values with *different lowercase* mean the significant difference at $P<0.05$ level

Table 1 Effects of exogenous H_2S on NPT and total GSH contents in cauliflower leaves under Pb stress

Treatments	NPT (nmol/g Fw)	Total GSH (nmol/g Fw)
CK	17.90 ± 1.22d	26.16 ± 5.93c
0.2 mM H_2S	38.47 ± 2.04c	30.85 ± 5.03c
0.3 mM H_2S	22.96 ± 3.89d	28.42 ± 6.20c
0.5 mM Pb	137.68 ± 11.44b	64.88 ± 5.84b
0.5 mM Pb + 0.2 mM H_2S	174.22 ± 10.95a	81.58 ± 6.24a
0.5 mM Pb + 0.3 mM H_2S	168.76 ± 16.18a	81.10 ± 8.60a

Data are presented as the mean ± SD ($n=4$). Values with *different lowercase* mean the significant difference at $P<0.05$ level

that low concentrations of NaHS had slight impacts on cauliflower whereas higher concentrations of 1.5–3.0 mM significantly inhibited seedling growth. It is consistent with the findings from Chen and others (2013a). So the effects of H_2S is concentration dependent. NaHS at 0.2 mM had the greatest effect on Pb detoxification in cauliflower.

Yang and others (2016) reported that PbAc inhibited six forest tree seed germination and the inhibitory effect order from strong to weak was RL, SL, GP, and SW. Similar results were also found in our study (Fig. 1). Roots are directly exposed in the soil or solution and accumulated the highest amounts of Pb compared to shoots or leaves (Ali and others 2014; Bharwana and others 2014; Hu and others 2015; Sidhu and others 2016). On the other hand, the root endodermis might act as a barrier to the absorption of Pb into the stele and transport to shoots (Reis and others 2015). When Pb entered the radicle cells, it bound

with nucleic acid, disrupted mitosis, and then influenced cell division and root elongation (Yang and others 2016). However, exogenous H_2S distinctly lowered the Pb concentration in roots and shoots (Ali and others 2014; Bharwana and others 2014). But the mechanism for preventing Pb uptake by intracellular H_2S is still unclear. The improved effect of H_2S on root morphology and cell structure, that is, a well-developed nucleus with continuous cell membrane, has been proven by Ali and others (2014) and this may be the crucial contribution to the enhanced root growth.

PbAc exposure significantly up-regulated ROS production both in vivo and in vitro (Dewanjee and others 2015; Dua and others 2016). In this investigation, the decreases in MDA, $O_2\cdot^-$, and H_2O_2 contents confirm the protective role of H_2S against Pb stress in cauliflower (Figs. 3, 4). It is in line with the findings in cotton and tall fescue under Pb stress (Bharwana and others 2014; Hu and others 2015). Similar results about H_2S were also found in plants under other heavy metal stresses (Chen and others 2013a; Mostofa and others 2015; Zhang and others 2008). Reduced oxidative damage under stresses was usually attributed to enhanced antioxidant enzyme activities stimulated by H_2S application (Bharwana and others 2014; Li and others 2014; Mostofa and others 2015; Sun and others 2013; Zhang and others 2010). In contrast, it should be note that the activities of SOD, CAT, POD, APX, and GR were not up-regulated by H_2S in this study (Fig. 5). The same phenomenon was also found by Chen and others (2013a), who described that in plants treated with 200 µM H_2S pre-application plus Al stress, the decreased SOD, APX, and POD activities matched the lower H_2O_2 level and reduced oxidative damage. The authors considered that H_2S diminished the need for antioxidant enzymes by keeping the ROS levels low in plant tissues. But there were no explanations about how this happened in plants. In the bacteria of *Shewanella oneidensis*, Wu and others (2015) reported that H_2S had no influence on SOD activity but depressed CAT and POD activities, suggesting that neither SOD nor CAT was the target of H_2S. So we speculated that H_2S might act as a ROS scavenger similar to NO (Chen and others 2015; Saxena and Shekhawat 2013; Singh and others 2009) or as a reductant (Kabil and others 2014), and therefore results in accelerated detoxification. It was proven by the application of antioxidant enzyme inhibitors and ROS scavengers.

DDC can bind and remove Cu (II) ions from Cu/Zn-SOD and inhibit its activity (Lushchak and others 2005). So when DDC was added to the solution treated by Pb, SOD activity declined (Fig. 5a) and $O_2\cdot^-$ was not rapidly broken down into H_2O_2 and kept at a high level (Fig. 4). Nevertheless, H_2S supplement effectively eliminated $O_2\cdot^-$ though SOD activity was maintained at a low level (Figs. 4, 5a). Moreover, AT is a special inhibitor of CAT and affects H_2O_2 decomposition (Han and others 2009). As the same trend, AT caused CAT activity inhibition and H_2O_2 accumulation, whereas H_2S helped in H_2O_2 scavenging by not up-regulating CAT activity (Figs. 4, 5b). Furthermore, Tiron and DMTU, specific traps for $O_2\cdot^-$ and H_2O_2, have been proven to prevent oxidative and DNA damage caused by Al, H_2O_2, or UVB (Chen and others 2009; Fang and others 2012; Murali Achary and Panda 2010; Sharma and Mishra 2006). In the present experiments, they also efficiently scavenged ROS induced by Pb stress and then reduced oxidative damage (Figs. 3, 4). And H_2S had a comparable property to eliminate ROS thoroughly just as Tiron and DMTU did. All these data demonstrated the role of H_2S in $O_2\cdot^-$ and H_2O_2 scavenging or reduction. Al-Magableh and others (2014) reported that NaHS not only directly scavenged $O_2\cdot^-$, but also suppressed vascular NADPH oxidase (Nox)-derived $O_2\cdot^-$ production in vitro. H_2S and HS^- can act as reductants by the single hydrogen atom 'H' or electron 'e' transfer and scavenge ROS and nitrogen species such as $O_2\cdot^-$, H_2O_2, peroxynitrite, and hypochlorous acid (Al-Magableh and others 2014; Li and Lancaster 2013). But this direct role is still in debate for its relatively lower concentration in vivo (Olson 2012; Xie and others 2016). So precise quantification and location of H_2S are urgently needed (Hancock and Whiteman 2016). Nevertheless, our experiments definitively confirmed the ROS eliminating capacity of H_2S. On the other hand, when H_2O_2 was scavenged by DMTU, the increased H_2S might act as a stress signal as H_2O_2 did.

Another antioxidant process for scavenging free radicals is via non-enzymatic antioxidants. NPT, including cysteine, GSH, and phytochelatins, plays important roles during heavy metal detoxification and GSH is the major component of NPT (Kotrba and others 2009). H_2S activated nuclear factor-erythroid 2-related factor 2 (Nrf2) to induce antioxidant response element (ARE)-dependent gene expression, such as the glutamate–cysteine ligase catalytic subunit (GCLM), glutamate–cysteine ligase catalytic modifier subunit (GCLC), and GR, and then increased the major intracellular antioxidant, Trx and GSH, to scavenge ROS directly (Xie and others 2016). GSH donates H^+ and e^- from its –SH groups of cysteine residues to ROS to neutralize them and then itself converts to GSSG (Dua and others 2016). And it was estimated that about 40% of the H_2S was converted to GSH (Li and others 2012b). Pb has a strong likeness toward the –SH group (Dua and others 2016). Therefore, the distinctly increased NPT and GSH efficiently enhanced the tolerance of cauliflower for Pb stress by scavenging ROS or chelating Pb directly. The antioxidant effect launched by H_2S is not via a single or simple way but is very complicated and a signaling pathway may also be involved (Bian and others 2016; Hancock and Whiteman 2016; Li and others 2016; Wang and others 2012; Xie and others 2016). So further investigations are

still needed for comprehensive elucidation of the protective mechanisms from H_2S.

In conclusion, 0.25 and 0.5 mM Pb stress significantly inhibited seed germination and seedling growth of cauliflower. However, applications of exogenous H_2S (NaHS) with suitable concentrations markedly increased leaf H_2S content and alleviated Pb phytotoxicity. Germination percentage, root length, shoot length, and fresh weight notably increased whereas MDA, $O_2\cdot^-$, and H_2O_2 production declined. H_2S prevented oxidative damage from Pb or Pb and antioxidant enzyme inhibitor (DDC or AT) dual stresses, and the protective role of H_2S was parallel to the ROS scavengers, Tiron, and DMTU. Moreover, H_2S elevated NPT and total GSH levels to chelate Pb or scavenge ROS directly. Our results demonstrated the strong protective and antioxidant role of H_2S.

Acknowledgements This work was supported by Taizhou University Scientific Research Project (2014PY024), Zhejiang Provincial Key Laboratory of Plant Evolutionary Ecology and Conservation open projects (EEC2014-08), and Taizhou Municipal Innovation Team and Key Laboratory Independently Design and Technology Projects (1403ky06).

Author Contribution ZC experiment design, result analysis and discussion, manuscript writing and revising. BFY experiment performance, manuscript editing. ZKH experiment guide, manuscript revising. JQZ, YZ, TTX experiment performance.

Compliance with Ethical Standards

Conflict of interest There is no conflict of interest and all authors have read and approved the final manuscript.

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