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REGULAR ARTICLE

Hydrogen sulfide enhances alfalfa (*Medicago sativa*) tolerance against salinity during seed germination by nitric oxide pathway

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Abstract
Aims and methods The molecular mechanisms and signal transduction pathways of hydrogen sulfide (H_2S) in plant biology are still unclear. Here, by using pharmacological and biochemical approaches, we report that H_2S promotes germination and alleviates salinity damage involving nitric oxide (NO) pathway.
Results Upon 100 mM NaCl treatment, both H_2S donor sodium hydrosulfide (NaHS) and NO donor sodium nitroprusside (SNP) at 100 μM could significantly attenuate the inhibition of alfalfa (*Medicago sativa*) seed germination and thereafter seedling growth inhibition. Meanwhile, the ratio of potassium (K) to sodium (Na) in the root parts was increased. Total, isozymatic activities or corresponding transcripts of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD), or ascorbate peroxidase (APX) were activated differentially, thus resulting in the alleviation of oxidative damage. The above protective roles of NaHS might be related to the induction of endogenous NO, because the addition of the specific scavenger of NO 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) reversed above effects. Meanwhile, NaHS-triggered NO production was confirmed.
Conclusions Our observations indicate that H_2S enhances plant responses against salinity stress by reducing oxidative damage, which might have a possible interaction with NO.

Keywords Hydrogen sulfide · *Medicago sativa* · Nitric oxide · Oxidative damage · Salinity stress · Seed germination

Abbreviations

APX	Ascorbate peroxidase
ASC	Ascorbic acid
CAT	Catalase
cPTIO	2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt
H_2S	Hydrogen sulfide
NaHS	Sodium hydrosulfide
NO	Nitric oxide
POD	Guaiacol peroxidase
SNP	Sodium nitroprusside

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SOD Superoxide dismutase
TBARS Thiobarbituric acid reactive substances

Introduction

In animals, the group of gaseous messengers continues to expand and now includes nitric oxide (NO), carbon monoxide (CO), hydrogen sulfide (H_2S), and several molecules from the broad category of reactive oxygen species (ROS). Like NO and CO, it has been confirmed that H_2S at physiologically relevant levels affects the structures and functions of the human body at molecular, cellular, tissue, and systemic levels. During the last decade, the development in the identification of H_2S as an important gasotransmitter becomes the focus of biology systems (Wang 2003; Lefer 2007; Baskar and Bian 2011). Meanwhile, recent studies confirmed that H_2S was able to induce adventitious rooting (Zhang et al. 2009a). The role of H_2S is also extended to the regulation of diverse aspects of plant responses against abiotic stresses. For example, it has been shown that H_2S plays a role in plant adaptive responses to boron (Wang et al. 2010), aluminum (Zhang et al. 2010b), and heavy metal exposure (Zhang et al. 2008), stomatal movement (Lisjak et al. 2010; García-Mata and Lamattina 2010), osmotic and drought stresses (Zhang et al. 2009b, 2010a). However, although H_2S plays a role in germination under stressful conditions (Zhang et al. 2008, 2010b), its precise role and the corresponding molecular mechanisms involved have not been fully elucidated.

It was well known that salt stress led to various negative influences on seed germination, seedling growth, and even plant productivity. Excess of salinity exerts adverse effects such as ion toxicity, osmotic strain, nutritional imbalances, and the inhibition of plant growth (Zhu 2001). Other important consequence of salinity stress is the overproduction of ROS, which shows toxicity to the metabolic functions in plants. In response to various stressful conditions, numerous studies have demonstrated that the modulation of seed germination of different plant species occurred when some growth hormones (auxin, GA, ABA, and ethylene), or other signaling substances (salicylic acid, hydrogen peroxide, NO, and CO, etc.) were applied (Arasimowicz and Floryszak-Wieczorek 2007; Wahid et al. 2007; Chen et al. 2008; Liu et al. 2010; Lee et al. 2010; Molassiotis et al. 2010). Therefore, knowledge of how these second messengers or modulators interact is crucial in gaining a better understanding of fundamental plant adaptive against various abiotic stresses.

Alfalfa (*Medicago sativa* L.) is the most widely grown perennial pasture in the world and its productivity in saline soils is considerably decreased due to improper nutrition of plants as well as osmotic and drought stresses. In this study, we carried out a set of germination assays to systematically examine the effect of H_2S on alfalfa seed germination. Germination parameters were measured either under normal growth conditions or in the presence of salinity stress. We found that exogenous H_2S donor sodium hydrosulfide (NaHS), could mimic the well-known NO donor sodium nitroprusside (SNP) effects on the alleviation of salinity-induced seed germination and seedling growth inhibition by the up-regulation of antioxidative system. Additionally, a possible cross-talk between H_2S and NO, which has been found in animals (Hosoki et al. 1997; Ondrias et al. 2008), was assessed by the addition of the specific NO scavenger, control chemicals of SNP and the detection of NO release upon various treatments.

Materials and methods

Chemicals

Sodium hydrosulfide (NaHS) and sodium nitroprusside (SNP), purchased from Sigma (St Louis, MO, USA), were used as the H_2S or HS^- and NO donor, respectively (Zhao et al. 2001; Bethke et al. 2006a, b; Xie et al. 2008; Wang et al. 2010). 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO) was used as the specific NO scavenger (Xuan et al. 2008; Liu et al. 2010). NO_2^-/NO_3^- and $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ were used as the degradation product of SNP and SNP analogue (Bethke et al. 2006a, b; Shi et al. 2007; Xie et al. 2008).

Plant material, growth condition and experimental design

Commercially available alfalfa (*Medicago sativa* L., Victoria) seeds were surface-sterilized with 5%

NaClO for 15 min, rinsed extensively in distilled water and then dried. These seeds were transferred to Petri dishes containing 4 ml of distilled water (Con), varying concentrations of NaCl (S) and NaHS, 100 μM SNP, 100/100 μM NO_2^-/NO_3^-, 100/100 μM $K_3Fe(CN)_6/K_4Fe(CN)_6$, 200 μM cPTIO, 100 μM $NaHSO_4$, 100 μM $NaHSO_3$, 100 μM Na_2S, 100 μM Na_2SO_4, 100 μM Na_2SO_3, and 100 μM NaAc alone, or containing two or three of the above chemicals, and kept at 25°C in a growth chamber in darkness for 2 or 4d of incubation. These solutions were renewed for every 2 days. All experiments were repeated for at least three times. After 2 or 4d of salinity treatment, the samples were harvested, growth parameters were determined, and material was frozen at −80°C for further analysis.

Germination and growth analysis

Germination tests were carried out on three replicates of 150 seeds each. There were 50 seeds in each Petri dish. Germination percentage or rate (%) was recorded every day for 2 or 4d, respectively, and seeds were considered to have germinated when the emerging shoot was approximately half the length of the seeds. Seed germination energy (GE,%)=(number of germinating seeds/number of total seeds per treatment after germination for 2 days)×100, according to the method described by Hu et al. (2004). Germination index (GI,%) was calculated as described in the Association of Official Seed Analysis (1983), using the following formulae:

$$GI = \frac{\text{No. of germinated seeds}}{\textit{Days of first count}} + \; + \frac{\text{No. of germinated seeds}}{\textit{Days of final count}}$$

The measurements of root length and shoot length were carried out at 4d after various treatments.

Determination of ion content

Na and K contents of root part were measured by an atomic emission spectrophotometer (TAS-986; Beijing Purkinje General Instrument Co., Ltd., Beijing, China).

Thiobarbituric acid reactive substances (TBARS) determination

Oxidative damage was estimated by measuring the concentration of TBARS as described by Han et al. (2008).

Antioxidant enzyme assays

Frozen alfalfa plants (approximately 200 mg) were homogenized in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone (PVP) for CAT, guaiacol POD, and SOD assay, or the combination with the addition of 1 mM ascorbic acid (ASC) in the case of APX assay. The homogenate was centrifuged at 12,000*g* in a rotor (model Avanti J-25, Beckman) for 20 min at 4°C and the supernatant was immediately desalted by Sephadex G-25 gel filtration to remove interfering materials and used as the crude enzyme extract.

Catalase (CAT) activity was spectrophotometrically measured by monitoring the consumption of H_2O_2 (ε= 39.4 M^{-1} cm^{-1}) at 240 nm for at least 3 min (Aebi 1984; Watanabe et al. 2003). Determination of guaiacol POD activity was performed by measuring the oxidation of guaiacol (ε=26.6 mM^{-1} cm^{-1}). The reaction mixture contained 50 mM potassium phosphate buffer (pH 6.4), 8 mM guaiacol, and 2.75 mM H_2O_2. The increase in absorbance was recorded at 470 nm within 2 min (linear phase) after the addition of H_2O_2 (Ruan et al. 2002). APX activity was measured by monitoring the decrease in absorbance at 290 nm as ASC was oxidized (ε=2.8 mM^{-1} cm^{-1}) for at least 1 min in 3 ml reaction mixture, as described by Nakano and Asada (1981). Total superoxide dismutase (SOD) activity was measured on the basis of its ability to inhibit the reduction of nitroblue tetrazolium (NBT) by superoxide anion generated by the riboflavin system under illumination. One unit of SOD (U) was defined as the amount of crude enzyme extract required to inhibit the reduction rate of NBT by 50% (Beauchamp and Fridovich 1971).

Gel electrophoresis

SOD isozymes were separated on native PAGE (5–20%). Proteins were electrophoresed for 20 h at 4°C at a constant voltage of 120 V in Tris-Gly buffer, pH 8.3. For each lane, 50 μg of protein extract was applied. SOD isozymatic activity on the gel was visualised by activity staining combined with the inhibitor test according to the procedure described by Beauchamp and Fridovich (1971) and Xie et al. (2008). All staining procedures were carried out at room temperature, and the reaction mixture contain-

ing gels was shaken at 75 rpm until the colorless bands of SOD activity in a purple-stained gel were visible. For the relative activity of different SOD isozymatic determination, gels were scanned in the transmission black-and-white mode and the intensity of bands was calculated by using the Quantity One v4.4.0 software (Bio-Rad, Hercules, California, USA). Then the band intensities of the individual isozymes were expressed as % of the control (Con) value.

Real-time quantitative RT-PCR analysis

Total RNA from 100 mg of fresh-weight alfalfa plants was isolated by grinding with mortar and pestle in liquid nitrogen until a fine powder appeared and using Trizol reagent (Invitrogen) according to the manufacturer's instructions. DNA-free total RNA (5 μg) from different treatments was used for first-strand cDNA synthesis in a 20-μL reaction volume containing 2.5 units of avian myeloblastosis virus reverse transcriptase XL (TaKaRa) and 2.5 μM random primer.

Real-time quantitative RT-PCR reactions were performed with Mastercycler® realplex^2 real-time PCR system (Eppendorf, Hamburg, Germany) using the SYBR® *Premix Ex Taq*™ (TaKaRa Bio Inc., China) according to the user manual. The cDNA was amplified using the following primers: *APX-1* (accession number DQ122791), forward 5′-TCCTCTTAT GCTCCGTTTG-3′ and reverse 5′-GTTCCACCCAG TAATCCCA-3′; *APX-2* (accession number AY054988), forward 5′-GTCCTTTCGGAACCATCAA-3′ and reverse 5′- GCAACAACACCAGCCAACT-3′; *Cu/Zn-SOD* (accession number AF056621), forward 5′-TAATTGCTGATGCCAACG-3′ and reverse 5′-ACCA CAGGCTAATCTTCCAC-3′; and *MSC27* (accession number X63872, Calderini et al. 2007), forward 5′-TGTCAGACTCTTACCCATAC-3′ and reverse 5′-CCTCATCTTCTCCACCTT-3′. The PCR program consisted of an initial denaturation and *Taq* activation step of 5 min at 95°C, followed by 40 cycles of 15 s at 95°C and 20 s at 50°C. A melting curve analysis was performed after every PCR reaction to confirm the accuracy of each amplified product. All reactions were set up in duplicate. Relative expression levels were presented as values relative to that of corresponding control sample at the indicated time, after normalization to *MSC27* transcript levels.

NO production determined by using greiss reagent

NO production was determined using the previous methods (Ding 1998; Xu et al. 2005; Zhou et al. 2005) with some modifications. Samples were ground in a mortar and pestle in 3 ml of 50 mM cool acetic acid buffer (pH 3.6, containing 4% zinc diacetate). The homogenates were centrifuged at 10 000*g* for 15 min at 4°C. The supernatant was collected. The pellet was washed by 1 ml of extraction buffer and centrifuged as before. The two supernatants were combined and 0.1 g of charcoal was added. After vortex and filtration, the filtrate was leached and collected. The mixture of 1 ml of filtrate and 1 ml of the Greiss reagent was incubated at room temperature for 30 min. Meanwhile, identical filtrate which was pretreated with 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (cPTIO), the specific scavenger of NO, for 15 min, was used as blanks. Absorbance was assayed at 540 nm. NO content was calculated by comparison to a standard curve of $NaNO_2$.

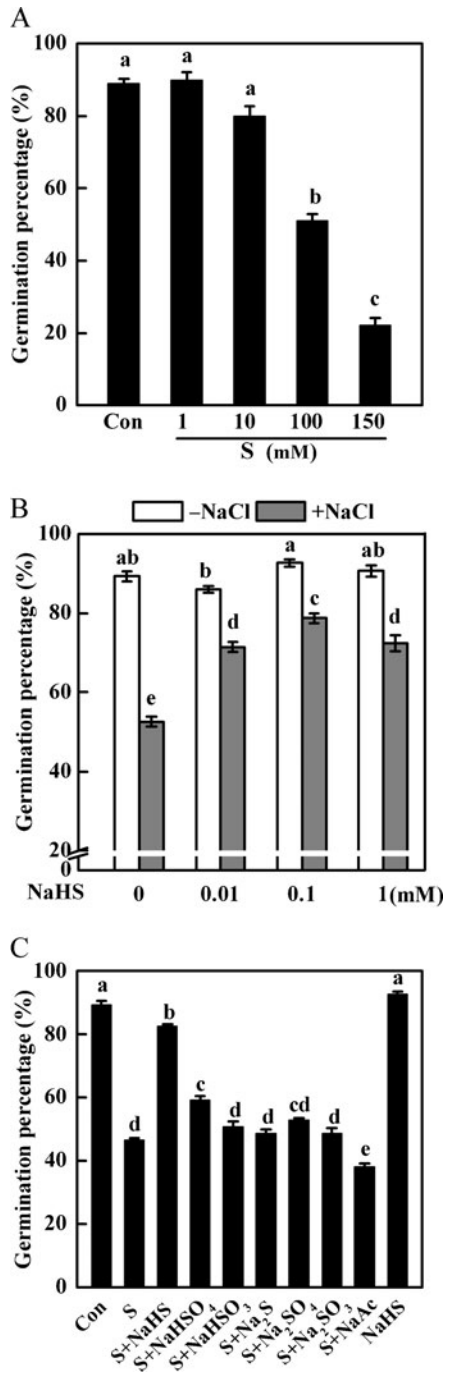
Statistical analysis

Where indicated, results are expressed as mean values ± SE of at least three independent experiments. For statistical analysis, Duncan's multiple test was used as appropriate, after testing for data normality. A value of $P<0.05$ was considered significant for mean differences.

Results

The NaCl-induced inhibition of seed germination and seedling growth restrain were rescued by H_2S

To assess the sensitivity of alfalfa seed germination to salinity stress, the effect of varying NaCl concentrations on seed germination was investigated. As expected, seed germination was decreased approximately in a dose-dependent fashion when exposed to 0–150 mM NaCl (Fig. 1a). Results further showed that in comparison with 100 mM NaCl-stressed alone sample, concentrations between 0.01 and 1.0 mM sodium hydrosulfide (NaHS), an effective H_2S or HS^- donor used both in animal and plant researches (Zhao et al. 2001; Kimura and Kimura 2004; Wang et al. 2010; Zhang et al. 2010a), alleviated alfalfa seed germination inhibition with a maximal response at



◀ **Fig. 1** Hydrogen sulfide (H_2S) or HS^-, but not other compounds derived from sodium hydrosulfide (NaHS), are contributed to the alleviation of seed germination inhibition caused by salinity stress for 2 days of incubation. Dose-dependent seed germination inhibition upon NaCl (S) treatments as indicated concentrations (**a**). Effects of NaHS at the indicated concentrations with or without 100 mM NaCl on the seed germination (**b**). Seeds were treated with either H_2O (Con), 100 mM NaCl (S), 100 μM NaHS, 100 μM $NaHSO_4$, 100 μM $NaHSO_3$,100 μM Na_2S, 100 μM Na_2SO_4, 100 μM Na_2SO_3, and 100 μM NaAc, or containing two of the above chemicals for 2 days of incubation (**c**). Values are the mean ± SE of at least three independent experiments. Different letters indicate significant differences ($P<0.05$) according to Duncan's multiple test

0.1 mM (Fig. 1b, Supplementary Information Fig. S1). Similarly, the cytoprotective roles of 0.1 mM NaHS were confirmed by the measurement of other germination and seedling growth parameters, such as germination energy (GE, 2d) and germination index (GI, 4d), root and shoot length (Table 1). However, no significant differences were observed when various concentrations of NaHS were used alone, comparing with the control sample. To verify the role of H_2S in the promotion of seed germination induced by NaHS, 0.1 mM Na_2S, Na_2SO_4, Na_2SO_3, $NaHSO_4$, $NaHSO_3$ and NaAc were used as the controls for Na^+- and sulfur-containing compounds. Interestingly, we noticed that these compounds were unable to exhibit the similar cytoprotective roles (Fig. 1c). Together, we suggested that H_2S or HS^-, rather than other compounds directly or indirectly derived from the decomposing of NaHS, were responsible for the promotive effects of NaHS on the amelioration of seed germination inhibition and seedling growth restrain triggered by NaCl exposure.

Above H_2S-triggered responses were sensitive to the specific scavenger of NO

In animals and recently in plants, most H_2S responses are similar to or mediated by NO (Ondrias et al. 2008; Zhang et al. 2009a; Wang et al. 2010). Endogenous or exogenous NO has been confirmed to induce seed germination under various abiotic stresses or reduce dormancy (Delledonne 2005). In the following experiments, our results confirmed that SNP, a well-known NO donor, was able to alleviate the salt-induced alfalfa seed germination and seedling growth inhibition ($P<0.05$; Table 1, Fig. 2a). To further confirm a specific role of NO in above responses, several controls such as NO_2^-/NO_3^- and $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ were employed. Contrary to the effects of SNP, no significant differences or negative responses were observed when NO_2^-/NO_3^- or $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ was added together with NaCl treatment (Table 1, Fig. 2a). Meanwhile, the control chemicals

Table 1 Effects of NaHS (100 μM), SNP (100 μM), cPTIO (200 μM), $K_3Fe(CN)_6/K_4Fe(CN)_6$ (100/100 μM), and NO_2^-/NO_3^- (100/100 μM) treatments on the inhibition of alfalfa seed germination, seedling root length and shoot length caused by 100 mM NaCl stress (S) for 4 days

Treatments	Germination rate (%)	Germination energy (%)	Germination index (%)	Root length (mm)	Shoot length (mm)
Con	96.3±1.5a	89.3±1.3ab	151.1±5.5ab	39.3±1.3a	25.0±0.9bc
S	78.5±3.4b	52.7±1.5e	53.4±1.2e	21.1±1.2 d	11.1±1.0f
S+NaHS	96.6±1.3a	78.7±1.2c	90.6±5.0c	31.1±1.3b	17.2±0.8 d
NaHS	97.2±1.0a	92.6±0.9a	163.1±8.5a	39.8±1.3a	25.3±0.4b
S+NaHS+cPTIO	83.6±2.9b	62.6±1.3 d	77.2±4.7 d	28.2±0.1c	15.6±0.4e
S+SNP	97.8±2.3a	85.3±1.2b	99.4±6.8c	32.5±0.9b	18.6±0.7 d
S+SNP+cPTIO	85.4±3.6b	58.2±1.6 de	66.7±3.7de	27.3±0.8c	14.5±0.8e
$S+K_3Fe(CN)_6/K_4Fe(CN)_6$	79.3±4.8b	54.9±4.0de	54.8±0.5e	22.8±0.6 d	11.6±0.5f
$S+NO_2^-/NO_3^-$	76.0±2.9b	57.2±1.1de	56.9±1.6e	21.3±1.6 d	9.5±0.6 g
SNP	96.5±0.4a	94.1±0.8a	162.2±2.4a	42.2±0.8a	27.8±0.8a
cPTIO	93.7±2.5a	86.0±0.8b	136.5±7.5b	39.0±1.1a	23.2±1.1c
$K_3Fe(CN)_6/K_4Fe(CN)_6$	92.0±1.2ab	85.0±2.9b	143.5±5.9b	34.8±0.4ab	24.5±0.4bc
NO_2^-/NO_3^-	88.0±2.3b	79.0±0.6c	128.8±2.4b	32.9±1.1b	24.3±0.7b

Values are the mean ± SE of three independent experiments. Germination energy was measured after 2 days of incubation. Different letters within columns indicate significant differences ($P<0.05$) according to Duncan's multiple test

exhibited the similar or negative response in comparison with the control sample (Table 1). To further assess whether endogenous NO was involved in responses induced by NaHS, the specific NO scavenger cPTIO was applied. As shown in Fig. 2a, the NaHS- and SNP-driven alleviation of salinity stress-induced alfalfa seedling growth inhibition was significantly prevented when cPTIO was added, correlating those data from seed germination parameters (Table 1, Supplementary Information Fig. S2). Meanwhile, plants exposed to cPTIO treatment alone exhibited a slight but no significant decrease of the above parameters in comparison with the NaCl-free control sample.

Reestablishment of ion homeostasis

The results of Fig. 2b illustrated that the inducible changes of K/Na ratio were observed in root tissues of NaHS- and SNP-treated samples, which is mainly caused by the accumulation of K^+ (data not shown). However, when exogenous cPTIO was added, above inducible responses were blocked significantly. Thus, the above results suggested that both NaHS and SNP treatment could enhance salinity tolerance by reestablishing ion homeostasis.

Lipid peroxidation

As expected (Bailly 2004), salinity stress brought about the obvious increase of TBARS level (Fig. 3). However, treatment with NaHS or SNP at 100 μM had the significant decreasing effects on salinity-induced accumulation of TBARS content. Generation of TBARS content was decreased by 59.4% and 50.7% in the alfalfa plants treated with NaHS or SNP plus NaCl compared with the NaCl-treated alone sample. By contrast, no significant difference was observed when NO_2^-/NO_3^- or $K_3Fe(CN)_6/K_4Fe(CN)_6$ was added together with NaCl treatment. Meanwhile, we demonstrated that the addition of cPTIO together with NaHS or SNP resulted in the obvious increases of TBARS content in salinity-stressed samples ($P<0.05$). In comparison with the control sample, the increase of TBARS content was also observed in cPTIO-treated alone sample.

Transcripts and activities of antioxidant enzymes

Salinity-induced oxidative stress in plants was associated with ROS overproduction (Hernandez et al. 2010; Miller et al. 2010). Thus, it was necessary to examine the antioxidative enzymes responsible for

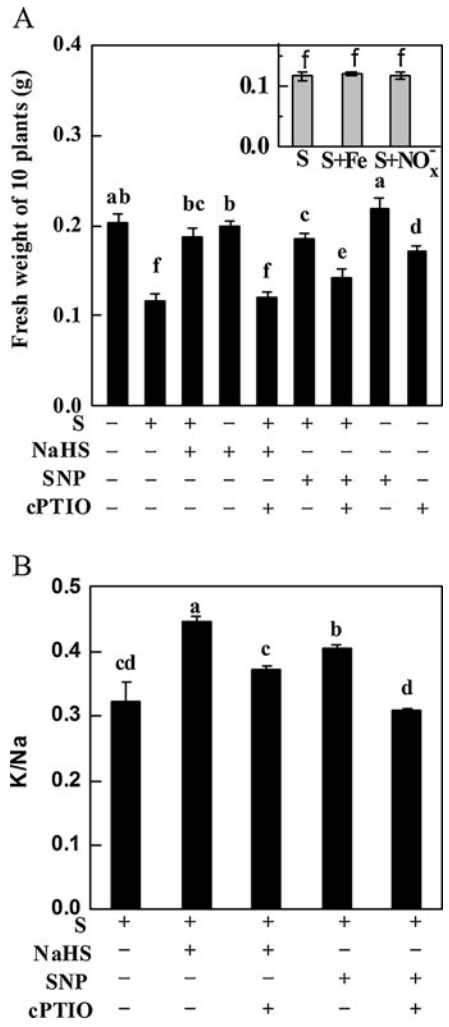


Fig. 2 Effects of cPTIO on NaHS- or SNP-induced the alleviation of growth constrains (**a**) and reestablishment of ion homeostasis (**b**) in alfalfa seedlings and seedling roots upon 100 mM NaCl exposure. The inset shows the effect of $K_3Fe(CN)_6/K_4Fe(CN)_6$ or NO_2^-/NO_3^- on NaCl-induced inhibition of seedling growth. Seeds were treated with either H_2O (Con), 100 mM NaCl (S), 100 μM NaHS, 100 μM SNP, 200 μM cPTIO, $K_3Fe(CN)_6/K_4Fe(CN)_6$ (100/100 μM, Fe), NO_2^-/NO_3^- (100/100 μM, NO_x^-), or containing two or three of the above chemicals for 4 days of incubation. Values are the mean ± SE of at least three independent experiments. Different letters indicate significant differences ($P<0.05$) according to Duncan's multiple test

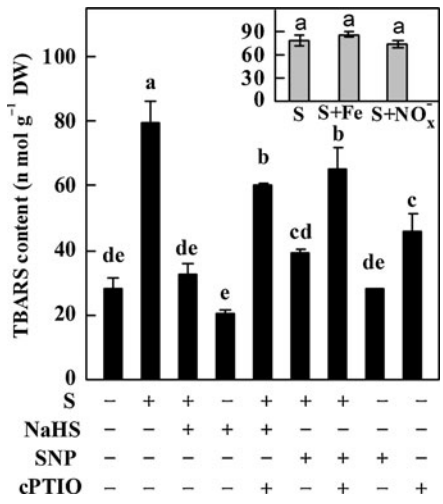


Fig. 3 Effects of cPTIO on NaHS- or SNP-induced the alleviation of lipid peroxidation in alfalfa seedlings upon 100 mM NaCl exposure. The inset shows the effect of $K_3Fe(CN)_6/K_4Fe(CN)_6$ or NO_2^-/NO_3^- on NaCl-induced lipid peroxidation. Seeds were treated with either H_2O (Con), 100 mM NaCl (S), 100 μM NaHS, 100 μM SNP, 200 μM cPTIO, $K_3Fe(CN)_6/K_4Fe(CN)_6$ (100/100 μM, Fe), NO_2^-/NO_3^- (100/100 μM, NO_x^-), or containing two or three of the above chemicals for 2 days of incubation. Values are the mean ± SE of at least three independent experiments. Different letters indicate significant differences ($P<0.05$) according to Duncan's multiple test

scavenging of ROS. The transcript levels of several antioxidant genes, such as *APX-1*, *APX-2*, and *Cu/Zn-SOD*, were down-regulated by salinity stress (Fig. 4). Similar decreased tendency was observed in total activities of APX, SOD, POD, and CAT (Fig. 5). Whereas, NaHS or SNP could significantly prevent salt-induced decreases of the transcripts and activities of these enzymes. Comparatively, the treatment in combined with cPTIO treatment produced a decrease tendency in the mRNA and activity levels compared with the values obtained when only NaHS or SNP was added together with NaCl. In addition, cPTIO alone produced the down-regulation of gene expression (except *Cu/Zn-SOD*) or activities (except CAT) of these antioxidant enzymes.

To confirm the responses of SOD, a native gradient polyacrylamide gel electrophoresis (PAGE, 5–20%) for SOD isozymatic analysis was performed. At least four SOD isoforms were detected in alfalfa plants (Fig. 6). As expected (Samis et al. 2001), isoforms I, II, III and IV, belonging to Mn-SOD, Fe-SOD, and Cu/Zn-SOD respectively (inhibitor test, data not shown), exhibited decreased activities in responses to salinity stress, whereas those with NaHS or SNP (especially) brought about the obvious induction. Interestingly, the application of cPTIO weaken the above responses, consisting with changes of lipid peroxidation (Fig. 3), ROS-scavenging enzymes or corresponding transcripts (Figs. 4, 5 and 6).

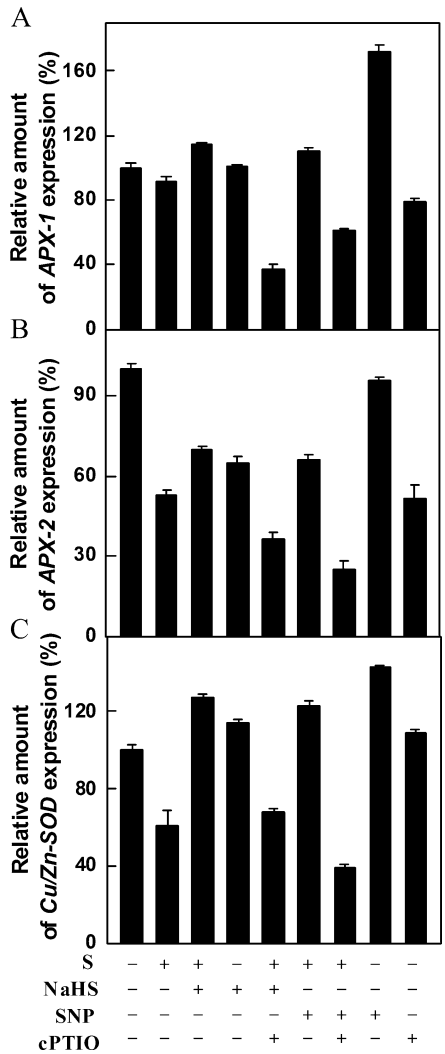


Fig. 4 Effects of cPTIO, NaHS and SNP on the transcripts of ascorbate peroxidase (APX, **a** and **b**), and Cu/Zn-superoxide dismutase (Cu/Zn-SOD, **c**) in alfalfa seedlings upon 100 mM NaCl exposure. Seeds were treated with either H_2O (Con), 100 mM NaCl (S), 100 μM NaHS, 100 μM SNP, 200 μM cPTIO, or containing two or three of the above chemicals for 24 h of incubation. The corresponding mRNA expression was analysed by real-time quantitative RT-PCR. The relative abundance of the corresponding genes is presented as values relative to the control sample. Values are the mean ± SE of at least three independent experiments

NO production triggered by NaHS

Above results suggested that NaHS-induced responses were sensitive to the scavenger of NO. To further verify whether above NaHS-driven responses were related to endogenous NO signal, we checked the endogenous NO level in our experimental conditions. As expected (Xie et al. 2008), NaCl stress for 24 h induced NO level moderately, as demonstrated by 21.5% increase in alfalfa seed tissues (Fig. 7). Meanwhile, NaHS (especially) and SNP treatment produced an increase of NO, being 26.9% and 8.1% higher than that of sample under salinity stress alone. By contrast, when cPTIO, the specific NO scavenger, was incubated with NaHS or SNP, the rate of NO generation was completely inhibited, reaching a lower level in comparison with those of the salt stressed alone sample. However, no significant difference was observed when cPTIO was applied alone in comparison with the control plants. Also, no significant changes of NO content was discovered when NO_2^-/NO_3^- or $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ was added together with NaCl treatment.

Discussion

H_2S is a water-soluble colorless molecule that easily penetrates biological membranes and exerts its effects independent of membrane receptors (Li et al. 2009). Until now, ample evidences have shown that H_2S acts as a signaling molecule in animals (Mancuso et al. 2010; Baskar and Bian 2011; Yong et al. 2011). In view of the fact that H_2S is produced in response to oxidative stress in yeast, it was deduced that H_2S functions as an antioxidant (Kimura and Kimura 2004). Given that plants can produce H_2S when subjected to relatively low concentrations of SO_2 and L-cysteine in the field and laboratory conditions (Hällgren and Fredriksson 1982; Sekiya et al. 1982), it is important to examine whether H_2S also plays a role in modulating of physiological processes in plants. More recently, investigations on the function of H_2S in plants have created some interesting discovers. For example, H_2S was shown to participate in the protection against copper toxicity (Zhang et al. 2008), stomatal movement (Lisjak et al. 2010; García-Mata and Lamattina 2010), adventitious rooting (Zhang et al. 2009a), and seed germination under osmotic stress by protecting these plants from oxidative damage (Zhang et al. 2009b). In the present study, we found that exposure of alfalfa seeds to increasing concentrations of NaCl treatment inhibited seed germination in a dose-dependent fashion (Fig. 1a). By using pharmacological approaches, we further demonstrated that the salinity-induced inhibi-

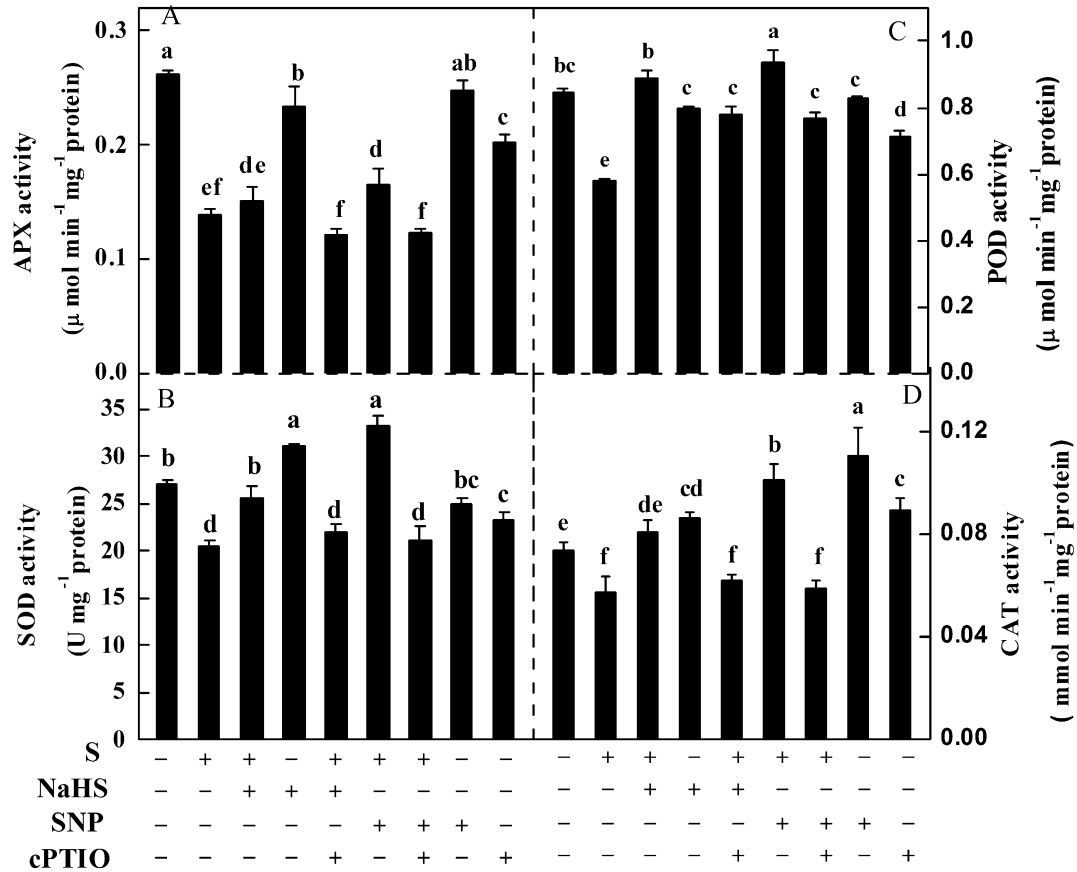


Fig. 5 Effects of cPTIO, NaHS and SNP on the activities of ascorbate peroxidase (APX, **a**), superoxide dismutase (SOD, **b**), guaiacol peroxidase (POD, **c**), and catalase (CAT, **d**) in alfalfa seedlings upon 100 mM NaCl exposure. Seeds were treated with either H_2O (Con), 100 mM NaCl (S), 100 μM NaHS, 100 μM SNP, 200 μM cPTIO, or containing two or three of the above chemicals for 24 h of incubation. Then, enzyme activities were determined. Values are the mean ± SE of at least three independent experiments. Different letters indicate significant differences ($P<0.05$) according to Duncan's multiple test

tory effect on seed germination and changes in seedling growth could be alleviated by NaHS (Figs. 1b, 2a, Table 1, Supplementary Information Fig. S1), the well-known H_2S and HS^- donor both in the animal and plant kingdoms (Zhao et al. 2001; Xie et al. 2008; Wang et al. 2010), by the regulation of antioxidant enzymes expression or establishment of ion homeostasis, all of which might be partially mediated by the NaHS-driven NO production. As expected (Delledonne 2005), similar cytoprotective roles of NO were confirmed by the use of NO donor SNP compared with no significant or slight negative responses conferred by its control chemicals (Table 1, Figs. 2a and 3).

In view of the fact that NaHS dissolves in water and dissociates to produce Na^+ and HS^-; HS^- associated with H^+ and produce H_2S, we further used other chemicals, such as S^{2-}, SO_4^{2-}, SO_3^{2-}, HSO_4^-, HSO_3^-, and Na^+, as controls for NaHS, but discovered that none were able to exhibit the similar cytoprotective role of NaHS in comparison with the NaCl stressed alone sample (Fig. 1c), Thus, above results confirmed that H_2S or HS^-, rather than other compounds derived from NaHS, play a role in the enhancement of salinity tolerance in alfalfa plants.

Ample evidence has confirmed that ROS overproduction in plants when upon various abiotic stresses, led to oxidative stress and cellular damage in plant tissues (Bailly 2004; Asada 2006; Hernandez et al. 2010; Miller et al. 2010). In response to salt stress, it is well established that antioxidant enzyme activities reveal stimulation, no-effect and suppression depending on different plant species, organ analyzed as well as the concentration and exposure duration of NaCl

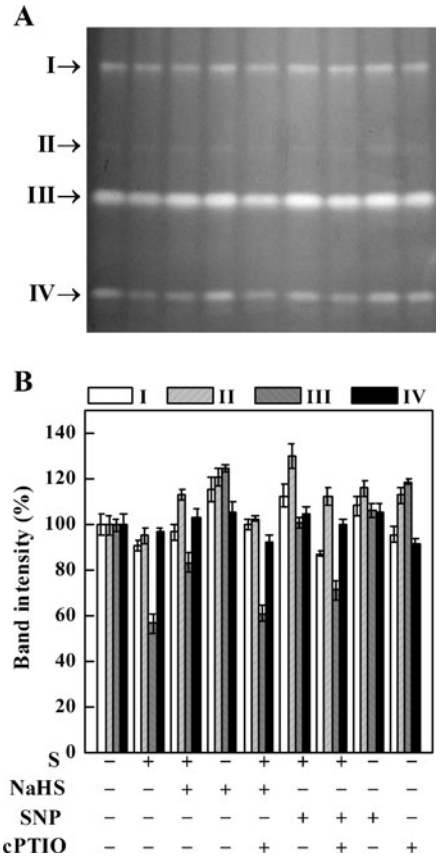


Fig. 6 Effects of cPTIO, NaHS and SNP on the isozymatic activities of superoxide dismutase (SOD) in alfalfa seedlings upon 100 mM NaCl exposure. Seeds were treated with either H_2O (Con), 100 mM NaCl (S), 100 μM NaHS, 100 μM SNP, 200 μM cPTIO, or containing two or three of the above chemicals. The gradient PAGE analysis of SOD isozymes (**a**) from salt-stressed alfalfa seeds for 24 h was determined. Meanwhile, relative activity of different SOD isozymes was also shown in (**b**). Band intensities of the individual isozymes were expressed as% of the control samples. Values are the mean ± SE of at least three independent experiments

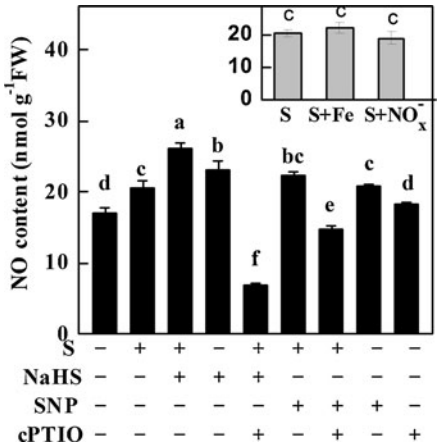


Fig. 7 NaHS-dependent NO generation. Seeds were treated with either H_2O (Con), 100 mM NaCl (S), 100 μM NaHS, 100 μM SNP, 200 μM cPTIO, $K_3Fe(CN)_6/K_4Fe(CN)_6$ (100/100 μM, Fe), NO_2^-/NO_3^- (100/100 μM, NO_x^-), or containing two or three of the above chemicals. NO contents of alfalfa seeds were detected by using Greiss reagent after 24 h of treatment. The inset shows the effect of $K_3Fe(CN)_6/K_4Fe(CN)_6$ or NO_2^-/NO_3^- on NaCl-induced NO generation. Values are the mean ± SE of at least three independent experiments. Different letters indicate significant differences ($P<0.05$) according to Duncan's multiple test

treatment (Yoshimura et al. 2000; Kacperska 2004; Hamed et al. 2007). Despite the lack of distinct patterns, above responses reflect the modified redox status of the plant cells induced by salinity stress. On the other hand, evidence has confirmed that plant tolerance to salinity stress is partially correlated with a more efficient antioxidant system (Miller et al. 2010). For example, the transformed tobacco over-expressing Arabidopsis *APX* showed more salinity and water stresses tolerance than the wild type (Badawi et al. 2004). In the subsequent experiment, our results further suggested that to cope with the elevation of lipid peroxidation triggered by salinity stress (Fig. 3), NaHS treatment differentially prevented the salt-induced decreases of *APX1/2* and *Cu/Zn-SOD* transcripts (Fig. 4) and APX, SOD, POD, and CAT total (Fig. 5) or SOD isozymatic (Fig. 6) activities in alfalfa seedlings upon salinity stress. We also noticed that there was a strong correlation among lipid peroxidation (Fig. 3), antioxidant enzyme and their expression (Figs. 4, 5 and 6), and corresponding responses of alfalfa seed germination and seedling growth parameters (Table 1, Fig. 2a) subjected to salinity stress. These results suggested that cytoprotective role of H_2S might be contributed to the increased activity of antioxidant enzymes (Figs. 5 and 6), which might be partially contributed to the up-regulation of their gene expression (Fig. 4), and thereafter decreased TBARS accumulation (Fig. 3). Above results correspondingly demonstrated the similar effects of H_2S in plants as that found in animal researches (Kimura and Kimura 2004). Other explanation of H_2S response might be attributed to its capability to exhibit antioxidant behavior (Li et al. 2009; Yonezawa et al. 2007). It was confirmed that H_2S was able to protect neurons against glutamate-mediated oxidative stress, or oxytosis, through the pleiotropic effects of maintaining the activities of γ-

glutamylcysteine synthetase (γ-GCS) and cysteine transport, leading to an increase in the well-known antioxidant glutathione (GSH) levels (Kimura and Kimura 2004). Similarly, the levels of cysteine and GSH were significantly increased by fumigation with H_2S in Arabidopsis (Riemenschneider et al. 2005).

When plants are exposed to NaCl, cellular ion homeostasis may be impaired. Thus, a high ratio of K/Na is a key factor of cellular adaptation to salt stress (Zhu 2003). In the subsequent test, we found that NaHS treatment resulted in a higher K/Na ratio in root parts of alfalfa seedlings 4 days after salt stress (Fig. 2b). This result illustrated that NaHS may help maintain the ion balance between K^+ and Na^+ in alfalfa seedlings upon salinity stress. Thus, ion homeostasis is reestablished so as to adapt to salt stress.

NO is a bioactive molecule mediating responses to biotic and abiotic stresses in the plant kingdom. It could induce germination instead of red light (Beligni and Lamattina 2000), affect growth and development of plant tissues (Durner and Klessig 1999), and be involved in the responses to drought stress, salinity exposure, and disease resistance (Delledonne 2005). In the subsequent experiment, another explanation for the above beneficial actions triggered by H_2S involved the NaHS-driven NO production (Fig. 7), which was consistent with the result reported by Jeong et al. (2006), showing that H_2S enhances NO production and inducible NO synthase (iNOS) expression by potentiating IL-1 ß-induced NF-κB activation through a mechanism involving extracellular signal-regulated kinase 1/2 (ERK1/2) signaling cascade in rat vascular smooth muscle cells (VSMCs). For example, the changes of germination parameters conferred by NaHS and SNP were partially reversed by treatment together with cPTIO (Table 1, Supplementary Information Fig. S2), a specific scavenger of NO, and reestablishment of ion homeostasis (Fig. 2b) and induction of antioxidative behaviors (Figs. 3, 4, 5 and 6) were also blocked. Taken together, all of these results suggested that endogenous NO might play a key role in H_2S-mediated cytoprotective roles, although we have not provided the potential source of NO triggered by NaHS treatment.

In conclusion, H_2S attenuated salinity-induced inhibition of alfalfa seed germination and seedling growth, and was partially due to the induction of antioxidant metabolism as well as the reestablishment of ion homeostasis. Interestingly, all these events might have a possible interaction with NO, one of the gaseoustransmitter recently confirmed in the plant kingdom (Delledonne 2005). Thus, this data further deepened our knowledge of the relationship between H_2S and NO in the enhancement of salinity tolerance in plants.

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