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Effects hydrogen sulfide on the antioxidant system and membrane stability in mitochondria of *Malus hupehensis* under NaCl stress

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Abstract

Salt stress is one of the most critical environmental factors limiting plant growth, and hydrogen sulfide (H₂S) can play a role in plant responses to this stress. To investigate the effects of H₂S on mitochondrial functions under salt stress, we treated *Malus hupehensis* Rehd. var. *pingyiensis* germinating seeds with an 85 mM NaCl solution with or without an H₂S donor sodium hydrosulfide (NaHS) and H₂S scavenger hypotaurine (HT). Then, hydrogen peroxide (H₂O₂) content and antioxidant enzyme activities were measured in mitochondria of seedling roots. Our results show that the application of 0.05 mM NaHS rescued an NaCl-induced inhibition of root elongation, decreased H₂O₂ content, and enhanced superoxide dismutase (SOD), guaiacol peroxidase (POD), and catalase (CAT) activities in the mitochondria compared to NaCl treatment alone. It was also found that 0.05 mM NaHS significantly decreased the mitochondrial permeability transition pore and increased mitochondrial membrane fluidity, mitochondrial membrane potential, and cytochrome *c/a* ratio under NaCl stress. However, 0.02 mM NaHS did not affect root growth, antioxidant enzyme activities, and mitochondrial function under NaCl stress, whereas high concentrations of NaHS (more than 0.2 mM) had a weaker or negative effects. Moreover, 15 μ M HT eliminated the beneficial effects of NaHS under NaCl stress. Our results suggest that H₂S protected plants against salt stress by decreasing H₂O₂ accumulation and by regulating membrane stability and antioxidant system in mitochondria.

Additional key words: apple, catalase, guaiacol peroxidase, membrane potential, root elongation, superoxide dismutase.

Introduction

Salinity is one of the most serious environmental and ecological problems and limits plant germination, growth, productivity, and survival, especially in saline zones (Ferreira-Silva *et al.* 2012, Zhou *et al.* 2015). High salinity affects water potential and ion balance at both the cellular level and the entire plant level, disrupting homeostasis and contributing to osmotic, ionic, and oxidative stresses in plants (Banaei-Asl *et al.* 2015). Most terrestrial plants are extremely vulnerable to rhizosphere salinization, especially during the seedling period (Muhammad *et al.* 2017). Plant roots are the organ that absorbs nutrients and water from the growth substrate and are strongly

influenced by salinity, particularly during the germination period.

Mitochondria are responsible for oxidation of sugars, fats, and amino acids, for ATP synthesis, and they regulate oxidative stress, apoptosis, *etc.* (Huang *et al.* 2014). Mitochondria are also the principal location of reactive oxygen species (ROS) production, particularly in non-photosynthetic cells such as root cells (Jacoby *et al.* 2012, Xia *et al.* 2015). Mitochondria are early targets of oxidative injuries, and environmental stress can cause mitochondrial damage and dysfunction (Ma *et al.* 2013). For example, nitrogen deficiency triggers accumulation of

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Abbreviations: ANS - 1-phenylamino-8-naphthalene sulfonic acid; BSA - bovine serum albumin; CAT - catalase; Cyt - cytochrome; EDTA - ethylenediamine tetraacetic acid; HEPES - N-2-hydroxyethylpiperazine-N-ethane-sulphonic acid; HT - hypotaurine; MDA - malondialdehyde; MPTP - mitochondrial permeability transition pore; NaSH - sodium hydrosulfide; PCD - programmed cell death; POD - peroxidase; ROS - reactive oxygen species; SOD - superoxide dismutase; $\Delta\psi_m$ - mitochondrial membrane potential.

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superoxide radical, hydrogen peroxide, and malondialdehyde (MDA) and increases mitochondrial lipid peroxidation under hypoxia stress (Sheng *et al.* 2009). The integrity of the mitochondrial ultrastructure decreases during ageing of oat seeds (Xia *et al.* 2015). Heat leads to the translocation of cytochrome (Cyt) *c* from mitochondria to the cytosol during programmed cell death in cucumber plants (Balk *et al.* 1999). Aluminum toxicity inhibits mitochondrial respiration, decreases ATP content, alters redox status, and destroys the internal structure, finally resulting in cell death in tobacco cells (Panda *et al.* 2008). Furthermore, it was reported that NaCl stress can also cause mitochondrial damage and dysfunction in plants. Stress caused by NaCl destroys the integrity of mitochondria, induces Cyt *c* release from mitochondria into the cytosol, and inhibits electron transport, which results in oxidative burst in mitochondria (Chen *et al.* 2009). Treatment with NaCl leads to decrease of mitochondrial membrane potential ($\Delta\psi_m$) and H_2O_2 content in roots of *Malus hupehensis* seedlings (Ma *et al.* 2010). In previous studies, most researchers focused on the relationship between stress and mitochondrial function to reveal the mechanisms of stress injury. However, studies investigating how to enhance resistance by preventing or alleviating the damage on mitochondria need to be solved.

Hydrogen sulfide (H_2S) is an endogenous gaseous signaling molecule discovered in recent years. It plays an important role in improving plant cell adaptability to environmental stresses such as cold, drought, salinity, and heavy metals (Jin *et al.* 2011, Chen *et al.* 2013, Bharwana *et al.* 2014, Shan *et al.* 2014, Deng *et al.* 2016, Liu *et al.* 2016, Du *et al.* 2017). Studies have shown that H_2S dramatically increases the survival rate of *Arabidopsis thaliana* seedlings (Jin *et al.* 2011) and delays the accumulation of MDA, H_2O_2 and superoxide radical in

soybean seedlings (Zhang *et al.* 2010a) under drought stress. The H_2S also counteracts chlorophyll loss in sweet potato leaves, alleviates oxidative damage against osmotic stress (Zhang *et al.* 2009), improves heat tolerance in tobacco cell suspension (Li *et al.* 2012, 2013), and alleviates root tip death induced by hypoxia in pea seedlings (Cheng *et al.* 2013). Furthermore, H_2S also plays an important role in plant responses to heavy metals including mercury (Chen *et al.* 2017), aluminum (Dawood *et al.* 2012, Chen *et al.* 2013), copper (Zhang *et al.* 2010b), boron (Wang *et al.* 2010), cadmium (Qiao *et al.* 2015, Tian *et al.* 2016), and zinc (Liu *et al.* 2016). It has been confirmed that H_2S plays a protective role against NaCl stress; for example, H_2S could attenuate the salt-induced inhibition of seed germination and seedling growth in alfalfa by reducing oxidative damage (Wang *et al.* 2012) and alleviates salt damage in maize leaves by enhancing ascorbate and glutathione metabolism (Shan *et al.* 2014). However, whether H_2S participates in regulation of mitochondrial antioxidant enzyme activities and mitochondrial function in plants under salt stress remains unknown.

Sodium hydrosulfide (NaHS), often used as an H_2S donor, and hypotaurine (HT), a scavenger of H_2S (Jin *et al.* 2011, Zhang *et al.* 2009, Li *et al.* 2012), were used in the current experiments. Root elongation, antioxidant enzyme activities, mitochondrial membrane permeability, membrane fluidity, membrane potential, and Cyt *c/a* ratio as affected by NaCl and NaHS were investigated in the roots of *Malus hupehensis* var. *pingyiensis*. The aim of the study was to elucidate the impact of H_2S on mitochondrial function in salt-stressed plants and to provide a new insight into the role of H_2S in mitochondrial responses to salt stress.

Materials and methods

Plants and treatments: *Malus hupehensis* Rehd. var. *pingyiensis* is native in Taiyi mountain areas in Shandong province of China, and it is used as an apple rootstock. Uniform seeds were collected from the experimental field of the Shandong Agriculture University in the middle of October at the ripening time. Since *M. hupehensis* has a high degree of apomixis, the seedlings show little differences, and they are a good material for experiments. The seeds were surface sterilized in 75 % (v/v) ethanol for 2 min and in a 5 % (m/v) sodium hypochlorite solution for an additional 10 min (Chen *et al.* 2013). After three washes with distilled water, seeds were immersed in distilled water at 25 °C for 12 h, and then mixed with sand for stratification and maintained at 4 °C in a refrigerator for five weeks. The germinated seeds were selected and thoroughly rinsed with distilled water. To avoid a drastic damage, the germinated seeds were sprayed with 50 mM NaCl and transferred to Petri dishes with double filter

paper pre-wetted with 50 mM NaCl. After 12 h, the pre-treated germinated seeds were transferred to Petri dishes with double filter paper pre-wetted with different solutions. To analyze the effects of H_2S , the H_2S donor NaHS and H_2S scavenger HT were added to an 85 mM NaCl solution yielding seven combinations: 1) control (CK), 2) 85 mM NaCl alone, 3) 0.02 mM NaHS + 85 mM NaCl, 4) 0.05 mM NaHS + 85 mM NaCl, 5) 0.10 mM NaHS + 85 mM NaCl, 6) 0.20 mM NaHS + 85 mM NaCl, and 7) 0.05 mM NaHS + 15 μ M HT + 85 mM NaCl. The control seeds were treated with distilled water. The germinated seeds were sprayed with these solutions twice a day. Cultures were maintained in a growth chamber at night/day temperatures of 22/25°C, a relative humidity of 70 - 80 %, a photon flux intensity of 170 μ mol m⁻² s⁻¹, and a 14-h photoperiod. Experiments were performed with a randomized complete block design, and every treatment was carried out with three replications, and every Petri dish

was considered as a replicate. Root elongation, membrane stability, and antioxidant system in mitochondria were measured after seeds were grown under varying treatments for 24, 48, and 72 h.

Root length was measured with a Vernier calliper before and after treatments. Root elongation (ΔL) was expressed as the change of the root length after treatment in comparison with that before the treatment. In addition, a relative root elongation was expressed as: $RRE [\%] = \Delta L (\text{treatment group}) / \Delta L (\text{control group}) \times 100$.

Isolation of mitochondria was performed using the methods described by Panda *et al.* (2008) with some modifications. Root samples (5 g) were thoroughly rinsed with distilled water and then homogenized with a buffer containing 400 mM mannitol, 50 mM, Tris-HCl, 10 mM EDTANa₂, 0.1 % (m/v) bovine serum albumin, and 0.05 % (v/v) β -mercaptoethanol, pH 7.4, in an ice bath. The homogenate was centrifuged at 600 g and 4 °C for 10 min, and the supernatant was collected and centrifuged again at 10 000 g and 4 °C for 10 min. The pellet containing mitochondria was washed with the same buffer and incubated on ice until used. The root mitochondria isolation was completed in 1 h, and the isolated mitochondria were used within 2 h. Mitochondria content was defined by their protein content, and protein content was determined by the Bradford (1976) method using BSA as a standard.

Measurement of H₂O₂ content and antioxidant enzyme activities: H₂O₂ content was determined with a titanium sulfate precipitation reaction (Panda *et al.* 2008). Isolated mitochondria were resuspended in a buffer (50 mM phosphate buffered saline, 5 mM EDTANa₂, pH 7.4). A reaction mixture contained 20 cm³ of the mitochondrial suspension, 880 cm³ of double-distilled water, and 100 cm³ of titanium sulfate and was then incubated at room temperature for 15 min. Absorbance at 415 nm was measured using a UV2550 (Shimadzu, Kyoto, Japan) spectrophotometer.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured according to Giannopolitis *et al.* (1977). One unit of SOD activity was measured as the amount of enzyme required to cause 50 % inhibition of the nitroblue tetrazolium (NBT) reduction measured at 560 nm. Peroxidase (POD, EC1.11.1.7) activity was assayed as described by Kochba *et al.* (1977) with some modifications. A 3 cm³ of reactant mixture contained 200 mM potassium phosphate buffer (pH 6.0), 0.017 % (v/v) H₂O₂, 0.038 % (v/v) guaiacol, and 0.1 cm³ of the resuspended mitochondria. Variations in absorbance were measured at 470 nm. Catalase (CAT, EC 1.11.1.6) activity was assayed according to Dhindsa *et al.* (1982).

Determination of mitochondrial permeability transition pore opening, membrane fluidity, and membrane potential: The mitochondrial inner membrane shows a high permeability for sucrose and mannitol because of the opening mitochondrial permeability transition pores (MPTPs), which leads to a decrease in absorbance at 540 nm. This characteristics was measured according to Hu *et al.* (2015). Separated and purified mitochondrial pellets were suspended in a cooled MPTP test buffer (200 mM mannitol, 70 mM sucrose, 5 mM HEPES; pH 7.2). After a 2-min incubation at 20 °C, absorbance at 540 nm was determined using a UV2550 (Shimadzu, Kyoto, Japan) spectrophotometer. Protein content determined by the Bradford method was adjusted to 0.3 mg cm⁻³.

Mitochondrial membrane fluidity was determined as described by Pan *et al.* (2016). The membrane surface was labeled with a fluorescent lipid probe 1-phenylamino-8-naphthalene sulfonic acid (ANS). Isolated mitochondria were suspended in a buffer (50 mM phosphate buffered saline, 5 mM EDTANa₂, pH 7.4). A 3 cm³ of the suspension was mixed with 0.3 mM mannitol (2.85 cm³) and 5 mM ANS (0.06 cm³) and after a 60-s incubation, fluorescence was measured three times at 5-s intervals using a fluorescence spectrofluorometer (F4500, Shimadzu).

Measurement of mitochondrial membrane potential ($\Delta\psi_m$) was performed according to Zhan *et al.* (2014) with slight modifications. Isolated mitochondria were suspended in a buffer (250 mM sucrose, 2 mM N-2-hydroxyethylpiperazine-N-ethane sulphonic acid (HEPES), 0.5 mM KH₂PO₄, 4.2 mM sodium succinic acid, pH 7.4) and then stained with 1 μ g cm⁻³ Rhodamine 123 in the dark at 25 °C for 30 min. The samples were centrifuged again at 10 000 g and 4 °C for 10 min and washed three times in the buffer indicated above. After resuspension, fluorescence of each sample was measured three times at 5-s intervals using a F4500 spectrofluorometer.

Detection of cytochrome *c/a* ratio: The Cyt *c/a* ratio was assessed as described by Tonshin *et al.* (2003). Isolated mitochondria were suspended in 0.2 % (m/v) BSA, and absorbances were measured at 550 nm and 630 nm. Cyt *c/a* was calculated by the ratio of absorbances at 550 and 630 nm.

Statistical analyses: Each treatment was performed with three replicates. Results are presented as means $\pm$ standard deviations (SDs). Two-way analysis of variance (ANOVA) was performed using the software Excel, SPSS 19.0, and SigmaPlot 10.5. Post hoc comparisons were performed using the Tukey least-significant difference (LSD) test at $\alpha = 0.05$.

Results

To assess the effects of NaCl stress on *M. hupehensis*, we measured root length after seed germination at 85 mM NaCl for 72 h (Fig. 1 Suppl.). The root elongation was significantly inhibited by this NaCl treatment (Fig. 1). Further, we found that 0.02, 0.05, or 0.1 mM NaHS positively affected root growth, and 0.05 mM NaHS treatment had the greatest effect. The relative root elongation increased by 29.46, 71.89, and 70.40 % at 24, 48, and 72 h, respectively, in comparison with NaCl alone (Fig. 1). However, a high NaHS concentration (0.2 mM) did not affect the root elongation under NaCl stress. The addition of HT to 0.05 mM NaHS treatment canceled the positive effect of NaHS on root elongation (Fig. 1).

Fig. 1. The effect of NaHS on relative root elongation of *Malus hupehensis* seedling roots under 85 mM NaCl stress. Germinated seeds were treated with NaHS or hypotaurine (HT) at the indicated concentrations. Means $\pm$ SDs, $n = 3$, different letters indicate significant differences between treatments (LSD test, $\alpha = 0.05$).

Hydrogen peroxide originates from electron leakage in the electron transport chain in mitochondria, and from the production by SOD (Ma *et al.* 2013). The results show that H_2O_2 content in mitochondria decreased substantially up to 48 h and then rapidly accumulated at 72 h of NaCl stress. The application of 0.05 mM NaHS had the greatest effect on preventing H_2O_2 accumulation in mitochondria, which decreased by 31.63 % at 24 h, 39.80 % at 48 h and 37.30 % at 72 h compared to treatment with NaCl alone. A low NaHS concentration (0.02 mM) did not decrease obviously the H_2O_2 content. However, at an NaHS concentration above 0.2 mM, no extra positive effect was evident indicating that a high concentration of NaHS would be toxic. Furthermore, addition of HT eliminated the positive effect of 0.05 mM NaHS in decreasing H_2O_2 content and resulted in similar effects as the NaCl treatment alone at 72 h (Fig. 2). These findings indicate that H_2S can reduce the accumulation of H_2O_2 in mitochondria under NaCl stress.

To investigate the effects of NaHS on the antioxidant

system in mitochondria under NaCl stress, the activities of SOD, POD, and CAT were measured. All the activities were stimulated in the early stage of the NaCl stress, however, with the extending exposure to NaCl stress, the activities declined significantly at 72 h (Fig. 3). The treatments with 0.02 - 0.10 mM NaHS could alleviate the NaCl-induced decrease in SOD, POD and CAT activities after 72 h, and the application with 0.05 mM NaHS showed the most significant effect. A high NaHS concentration (0.2 mM) did not notably affect enzyme activities compared to the treatment by NaCl alone. However, when H_2S was scavenged with HT, the positive effects of 0.05 mM NaHS on the activities of SOD, POD and CAT in mitochondria were cancelled.

To investigate whether H_2S has an important

Fig. 2. The effect of NaHS on H_2O_2 content in mitochondria of *Malus hupehensis* seedling roots under 85 mM NaCl stress. Germinated seeds were treated with NaHS or hypotaurine (HT) at the indicated concentrations. Means $\pm$ SDs, $n = 3$, different letters indicate significant differences between treatments (LSD test, $\alpha = 0.05$).

role in membrane stability in mitochondria, membrane fluidity, MPTP, $\Delta\psi_m$, and Cyt *c/a* ratio were measured under NaCl stress. The fluorescence anisotropy of ANS was used to reflect membrane surface layer fluidity, with larger fluorescent values indicating lower membrane surface fluidity. The opening of MPTPs leads to a decrease in absorbance at 540 nm (Pan *et al.* 2016). Exposure to 85 mM NaCl significantly increased fluorescence of ANS and decreased absorbance at 540 nm from 0 h to 72 h compared to the controls (Fig. 4A,B). The application of 0.02 or 0.05 mM NaHS significantly decreased fluorescence of ANS from 24 h to 72 h compared to treatment with NaCl alone (Fig. 4A). We found that 0.05 mM NaHS had the greatest effect on mitochondrial membrane fluidity under NaCl stress resulting in a decreased fluorescence of ANS by 29.37, 42.10, and 36.42 % at 24, 48, and 72 h, respectively. The application of 0.02, 0.05, or 0.1 mM NaHS increased absorbance at 540 nm under NaCl treatment, and 0.05 mM

NaHS had the greatest effect on inhibition of MPTP opening and significantly increased absorbance at 540 nm by 19.95, 44.79, and 129.68 % at 24, 48, and 72 h, respectively, compared to treatment with NaCl alone (Fig. 4B). However, concentrations of NaHS higher than 0.2 mM had no effects or showed negative effects on mitochondrial membrane fluidity and MPTP opening under NaCl stress. Moreover, the effects of 0.05 NaHS on mitochondrial membrane fluidity and MPTP opening were reduced following the application of HT.

There were no obvious changes in mitochondrial

Fig. 3. The effect of NaHS on superoxide dismutase (SOD, A), peroxidase (POD, B), and catalase (CAT, C) activities in mitochondria of *Malus hupehensis* seedling roots under 85 mM NaCl stress. Germinated seeds were treated with NaHS or hypotaurine (HT) at the indicated concentrations. Means $\pm$ SDs, $n = 3$, different letters indicate significant differences between treatments (LSD test, $\alpha = 0.05$).

membrane potential and Cyt *c/a* ratio under control treatment from 0 to 72 h. The NaCl stress significantly decreased $\Delta\psi_m$ and Cyt *c/a* from 24 to 72 h compared to values in the controls (Fig. 4C,D). The application

Fig. 4. The effect of NaHS on membrane fluidity (A), mitochondrial permeability transition pore (MPTP, B), membrane potential (C), and Cyt *c/a* ratio (D) in mitochondria of *Malus hupehensis* seedling roots under 85 mM NaCl stress. Germinated seeds were treated with NaHS or hypotaurine (HT) at the indicated concentrations. Means $\pm$ SDs, $n = 3$, different letters indicate significant differences between treatments (LSD test, $\alpha = 0.05$).

of 0.05 mM NaHS significantly increased $\Delta\psi_m$ and Cyt *c/a* from 24 h to 72 h compared to the treatment with NaCl alone and other NaHS treatments. The $\Delta\psi_m$ and Cyt *c/a* values at 0.05 mM NaHS increased of 42.31, 44.05, and 110.13 %; and 21.34, 30.31, and 95.20 % at 24, 48, and 72 h, respectively, compared to treatment with NaCl alone. In addition, the effects of 0.01 mM and 0.02 NaHS were time-dependent; germinated seeds pre-treated with

significant change in $\Delta\psi_m$ at 72 h and 48 h of treatment, respectively, compared to treatment with NaCl alone. In addition, 0.1 mM NaHS exhibited a significant effect on Cyt *c/a* only at 48 h of treatment. A higher concentration of NaHS (0.2 mM) showed no effects or negative effects on mitochondrial membrane potential and Cyt *c/a* under NaCl stress. Moreover, the effects of 0.05 NaHS were alleviated when HT was added.

Discussion

The mitochondrial electron transport chain is a major site of ROS formation, especially in non-photosynthetic cells of root tissues (Amirsadeghi *et al.* 2006), and it may participate in oxidative burst in plants (Huang *et al.* 2014). Environmental stresses, such as salinity, result in the overproduction of ROS in plants (Zhang *et al.* 2007, Huang *et al.* 2014). In the present study, inhibited root elongation, decreased antioxidant system and membrane stability in mitochondria were observed in the seedling roots of *M. hupehensis* in response to NaCl stress. However, under short stress conditions, the low H₂O₂ content observed in mitochondria treated with 0.05 mM NaHS was probably due to the stimulation of antioxidative enzymes. This was proved by the increase in SOD, POD, and CAT activities in mitochondria after 24 to 48 h of NaCl or NaCl + NaHS treatments. In our study, H₂S alleviated the NaCl caused oxidative stress by increasing activities of ROS scavenging enzymes and also played an important role in alleviating NaCl-induced inhibition of root elongation. Similar results were also reported when the role of H₂S in alleviating the toxicity of drought and heavy metals was investigated (Zhang *et al.* 2010a,b). It has been elaborated that plants have two ways to defense against ROS accumulation (Chongchatuporn *et al.* 2013), the first way is antioxidative enzymes, such as SOD, POD, and CAT, and the second way is non-enzymatic compounds, such as ascorbate and glutathione, so, it is necessary to further investigate the effects of H₂S on non-enzymatic antioxidants in mitochondria under NaCl stress. However, with a more severe stress, the scavenging system may become saturated by the increased rate of ROS production, ultimately leading to excessive ROS production (Zhang *et al.* 2010b). In this study, with an increased treatment time, SOD, POD, and CAT activities decreased indicating that the antioxidant defence system failed to completely scavenge excessive ROS, and so H₂O₂ content drastically increased at 72 h (Figs. 2 and 3). An increased ROS amount induces sustained MPTP opening (Szabo and Zoratti 2014) and results in ROS burst in a phenomenon known as ROS-induced ROS release, which eventually leads to a severe mitochondrial damage or cell death (Zorov *et al.* 2014), followed by more Cyt *c* release. The release of Cyt *c* from mitochondria could disrupt the respiratory electron transport chain, and this phenomenon

can increase ROS content, which in turn affects Cyt *c* release (Wang *et al.* 2014). Furthermore, excessive ROS could directly interact with mitochondrial proteins and lipids, causing their dysfunction and decreasing $\Delta\psi_m$ and mitochondrial membrane fluidity (Huang *et al.* 2014). The major site of ROS formation in mitochondria lies in the electron transport chain, especially at Complex I and Complex III in state 4 mitochondrial respiration (Chen *et al.* 2003). However, state 4 mitochondrial respiration is regulated by $\Delta\psi_m$; thus, a $\Delta\psi_m$ drop increases electron leakage (Ichas and Mazat 1998), which ultimately led to excessive ROS generation at 72 h and further to MPTP opening, decreased mitochondrial membrane fluidity, and loss of $\Delta\psi_m$. These changes resulted in mitochondrial swelling and rupture of the outer mitochondrial membrane and could explain the inhibition of root elongation under NaCl stress. The integrity of mitochondria decreases and release of cyt *c* has also been observed in rice under salt stress (Chen *et al.* 2009).

Hydrogen sulfide, the third endogenous gaseous transmitter after nitric oxide and carbon monoxide, was proved to play a role in alleviating biotic and abiotic stresses (Qian *et al.* 2014). Previous studies showed that H₂S is involved in responses to salt stress by alleviating an NaCl-induced increase of MDA content and electrolyte leakage, and by regulating ascorbate and glutathione metabolism in maize (Shan *et al.* 2014). Lai *et al.* (2014) also found that endogenous H₂S enhances NaCl tolerance by maintaining K⁺/Na⁺ homeostasis through preventing the NaCl-triggered K⁺ efflux, and modulated several genes/activities of antioxidant defence enzymes in alfalfa seedlings. In our study, we used 0, 0.02, 0.05, 0.1, and 0.2 mM NaHS, and we found that 0.05 mM NaHS treatment significantly improved SOD, POD, and CAT activities and decreased H₂O₂ content in mitochondria under NaCl stress, even after long-term salt stress treatment (Fig. 2,3), and these results are in agreement with those of Shan *et al.* (2014) and Lai *et al.* (2014). However, little information is known about the role of H₂S in membrane stability in mitochondria. In our study, it was proved that 0.05 mM NaHS could significantly inhibit MPTP opening and improve mitochondrial membrane fluidity under salt stress. Moreover, $\Delta\psi_m$ and Cyt *c/a* also increased when plants were treated with NaHS, indicating the protective

role of H₂S against the salt-induced damage of mitochondrial membranes in roots (Fig. 4). Since increasing evidences indicate that mitochondria play a pivotal role in programmed cell death (PCD) in plants, and ROS burst, MPTP opening, and $\Delta\psi_m$ loss are early markers of PCD induced by various stimuli (Yao *et al.* 2004). We suspected that H₂S also participated in PCD in the plants. Moreover, H₂S can eliminate oxygen radicals by restoring the disulphide bond. In addition, it has been proved in animals that sulfhydryl (-SH) can combine with harmful substances such as oxygen free radicals, electrophilic bases and metal ions to reduce cell damage, as well as to enhance cell recovery (Wang *et al.* 2014).

Numerous results demonstrated that exogenous H₂S participates in plant adaptive responses to multiple abiotic stresses *via* stimulation of antioxidative enzymes (Chen *et al.* 2013) or non-enzymatic antioxidants (Shan *et al.* 2014), however, the molecular mechanisms of H₂S signaling are still limited. As H₂S can interact with the sulfhydryl groups of selective proteins, yielding a hydropersulfide moiety (-SSH) in a process termed S-sulfhydration, more and more evidence shows that S-sulfhydration is an important redox signalling

mechanism (Kabil *et al.* 2010, Yang *et al.* 2013) which has been proved in animals. Krishnan *et al.* (2011) found that H₂S can inhibit PTP1B, the prototypic member of protein tyrosine phosphatases (PTPs) enzyme family, *via* sulfhydration of the active site Cys in PTP1B in cells required the presence of cystathionine- γ -lyase (CSE), a critical enzyme in H₂S production, and resulted in inhibition of phosphatase activity in endoplasmic reticulum stress of mouse. Yang *et al.* (2013) found that H₂S protects cells against senescence *via* S-sulfhydration of Kelch-like ECH-associated protein 1 (Keap1) and then activates nuclear factor-E2-related factor2 (Nrf2) to respond to oxidative stress in mouse. In our study, the results show that exogenous H₂S treatment can reduce the opening of the MPTP under NaCl stress, maybe also due to that H₂S can maintain the original structure of MPTP *via* S-sulfhydration. Liu *et al.* (2015) also suggested that H₂S participates in responses to low temperature, heat, salt, osmotic, and UV stresses by mediating protein S-sulfhydration in foxtail millet. However, knowledge of the H₂S-induced S-sulfhydration in the response to stress in plants require further investigation.

Conclusions

In this study, H₂S played an important role in alleviating the NaCl-induced inhibition of root elongation in *M. hupehensis* Rehd. var. *pingyiensis*. However, the effects of H₂S were dependent on its concentration. Our results demonstrate that 0.05 mM NaHS showed the optimal effects and strongly enhanced the antioxidant

system and membrane stability in mitochondria, as evidenced by increased SOD, POD and CAT activities, decreased endogenous H₂O₂ content, decreased mitochondrial membrane permeability, and increased mitochondrial membrane fluidity, membrane potential, and Cyt *c/a* ratio under NaCl stress.

References

- Amirsadeghi, S., Robson, C.A., McDonald, A.E., Vanlerberghe, G.C.: Changes in plant mitochondrial electron transport alter cellular levels of reactive oxygen species and susceptibility to cell death signaling molecules. - *Plant Cell Physiol.* **47**: 1509-1519, 2006.
- Balk, J., Leaver, C.J., McCabe, P.F.: Translocation of cytochrome *c* from the mitochondria to the cytosol occurs during heat-induced programmed cell death in cucumber plants. - *FEBS Lett.* **463**: 151-154, 1999.
- Banaei-Asl, F., Bandehagh, A., Uliaei, E.D., Farajzadeh, D., Sakata, K., Mustafa, G., Komatsu, S.: Proteomic analysis of canola root inoculated with bacteria under salt stress. - *J. Proteomics* **124**: 88-111, 2015.
- Bharwana, S.A., Ali, S., Farooq, M.A., Ali, B., Iqbal, N., Abbas, F., Ahmad, M.S.A.: Hydrogen sulfide ameliorates lead-induced morphological, photosynthetic, oxidative damages and biochemical changes in cotton. - *Environ. Sci. Pollut. Res. Int.* **21**: 717-731, 2014.
- Bradford, M.M.: A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye-binding. - *Anal. Biochem.* **72**: 248-254, 1976.
- Chen, J., Wang, W.H., Wu, F.H., You, C.Y., Liu, T.W., Dong, X.J.: Hydrogen sulfide alleviates aluminum toxicity in barley seedlings. - *Plant Soil* **362**: 301-318, 2013.
- Chen, Q., Vazquez, E.J., Moghaddas, S.: Production of reactive oxygen species by mitochondria: central role of complex III. - *J. Biol. Chem.* **278**: 36027-36031, 2003.
- Chen, X., Wang, Y., Li, J.Y., Jiang, A.L., Cheng, Y.W., Zhang, W.: Mitochondrial proteome during salt stress-induced programmed cell death in rice. - *Plant Physiol. Biochem.* **47**: 407-415, 2009.
- Chen, Z., Chen, M., Jiang, M.: Hydrogen sulfide alleviates mercury toxicity by sequestering it in roots or regulating reactive oxygen species productions in rice seedlings. - *Plant Physiol. Biochem.* **111**: 179-192, 2017.
- Cheng, W., Zhang, L., Jiao, C.J., Su, M., Yang, T., Zhou, L.N., Peng, R.Y., Wang, R.R., Wang, C.Y.: Hydrogen sulfide alleviates hypoxia-induced root tip death in *Pisum sativum*. - *Plant Physiol. Biochem.* **70**: 278-286, 2013.
- Chongchatuporn, U., Ketsa, S., Van Doorn, W.G.: Chilling injury in mango (*Mangifera indica*) fruit peel: relationship with ascorbic acid concentrations and antioxidant enzyme activities. - *Postharvest Biol. Technol.* **86**: 409-417, 2013.
- Dawood, M., Cao, F., Jahangir, M.M., Zhang, G., Wu, F.:

- Alleviation of aluminum toxicity by hydrogen sulfide is related to elevated ATPase, and suppressed aluminum uptake and oxidative stress in barley. - J. Hazard. Mater. **209-210**: 121-128, 2012.
- Deng, Y.Q., Bao, J., Yuan, F., Liang, X., Feng, Z.T., Wang, B.S.: Exogenous hydrogen sulfide alleviates salt stress in wheat seedlings by decreasing Na⁺ content. - Plant Growth Regul. - **79**: 391-399, 2016.
- Dhindsa, R.S., Plumb-Dhindsa, P., Thorpe, T.A.: Leaf senescence correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase. - J. exp. Bot. **32**: 93-101, 1982.
- Du, X.Z., Jin, Z.P., Liu, D.M., Yang, G.D., Pei, Y.X.: Hydrogen sulfide alleviates the cold stress through MPK4 in *Arabidopsis thaliana*. - Plant Physiol. Biochem. **120**: 112-119, 2017.
- Ferreira-Silva, S.L., Voigt, E.L., Silva, E.N., Maia, J. M., Aragão, T.C.R., Silveira, J.A.G.: Partial oxidative protection by enzymatic and non-enzymatic components in cashew leaves under high salinity. - Biol. Plant. **56**: 172-176, 2012.
- Gionannopolitis, C.N., Ries, S.K.: Purification and quantitative relationship with water-soluble protein in seedling. - Plant Physiol. **59**: 315-318, 1977.
- Hu, Z.G., Zhou, L., Ding, S.Z.: Effects of aerobic training to exhaustive exercise rat mitochondrial permeability transition pore. - J. Shenyang Sport Univ. **34**: 64-67, 2015.
- Huang, W.J., Yang, X.D., Yao, S.C., Thet, L.O., He, H.Y., Wang, A.Q., Li, C.Z., He, L.F.: Reactive oxygen species burst induced by aluminum stress triggers mitochondria-dependent programmed cell death in peanut root tip cells. - Plant Physiol. Biochem. **82**: 76-84, 2014.
- Ichas, F., Mazat, J.P.: From calcium signaling to cell death: two conformation for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. - Biochim. Biophys. Acta **1366**: 33-50, 1998.
- Jacoby, R.P., Li, L., Huang, S., Lee, C.P., Millar, A.H., Taylor, N.L.: Mitochondrial composition, function and stress response in plants. - J. integr. Plant Biol. **54**: 887-906, 2012.
- Jin, Z.P., Shen, J.J., Qiao, Z.J., Yang, G.D., Wang, R., Pei, Y. X.: Hydrogen sulfide improves drought resistance in *Arabidopsis thaliana*. - Biochem. biophys. Res. Co. **414**: 481-486, 2011.
- Kabil, O., Banerjee, R.: Redox biochemistry of hydrogen sulfide. - J. biol. Chem. **285**: 21903-21907, 2010.
- Kochba, J., Lavee, S., Spiegel-Roy, P.: Differences in peroxidase activity and isoenzymes in embryogenic and non-embryogenic 'Shamouti' orange ovular callus lines. - Plant Cell Physiol. **18**: 463-467, 1977.
- Krishnan, N., Fu, C.X., Pappin, D.J., Tonks, N.K.: H₂S-induced sulfhydration of the phosphatase PTP1B and its role in the endoplasmic reticulum stress response. - Sci. Signal. **4**: ra86, 2011.
- Lai, D.W., Mao, Y., Zhou, H., Li, F., Wu, M.Z., Zhang, J., He, Z.Y., Cui, W.T., Xie, Y.J.: Endogenous hydrogen sulfide enhances salt tolerance by coupling the reestablishment of redox homeostasis and preventing salt-induced K⁺ loss in seedlings of *Medicago sativa*. - Plant Sci. **225**: 117-129, 2014.
- Li, Z.G., Ding, X.J., Du, P.F.: Hydrogen sulfide donor sodium hydrosulfide improved heat tolerance in maize and involvement of proline. - Plant Physiol. **170**: 741-747, 2013.
- Li, Z.G., Gong, M., Xie, H., Yang, L., Li, J.: Hydrogen sulfide donor sodium hydrosulfide-induced heat tolerance in tobacco (*Nicotiana tabacum* L.) suspension cultured cells and involvement of Ca²⁺ and calmodulin. - Plant Sci. **185/186**: 185-189, 2012.
- Liu, X., Chen, J., Wang, G.H., Wang, W.H., Shen, Z.J., Luo, M.R., Gao, G.F., Simon, M., Ghoto, K., Zheng, H.L.: Hydrogen sulfide alleviates zinc toxicity by reducing zinc uptake and regulating genes expression of antioxidative enzymes and metallothioneins in roots of the cadmium/zinc hyperaccumulator *Solanum nigrum* L. - Plant Soil **400**: 177-192, 2016.
- Liu, Z.Q., Pei, Y.X., Fang, H.H., Tian, B.H.: [H₂S regulates foxtail millet responding to stress by protein S-sulfhydration.] - Chin. J. Biochem. Mol. Biol. **31**: 1085-1091, 2015. [In Chin., ab: E]
- Ma, H.Y., Lv, D.G., Liu, G.C.: Mitochondrial response in the apical and lateral flower buds of the Hanfu apple to cold stress during the dormancy stage. - Acta ecol. Sin. **33**: 52-58, 2013.
- Ma, H.Y., Lv, D.G., Yang H.Q.: Characteristics of mitochondria and cell death in roots of *Malus hupehensis* var. *pingyiensis* under NaCl stress. - Chin. J. Plant Ecol. **34**: 1448-1453, 2010.
- Muhammad, F., Nirmali, G., Mubshar, H., Sharmistha, B., Sreyashi, P., Nandita, B., Hussein, M.M., Salem, S.A., Kadambot, H.M.S.: Effects, tolerance mechanisms and management of salt stress in grain legumes. - Plant Physiol. Biochem. **118**: 199-217, 2017.
- Pan, X.B., Xiang, L.X., Hu, X.H., Ren, W.Q., Zhang, L., Ni, X.X.: Effects of exogenous spermidine on mitochondrial function of tomato seedling roots under salinity-alkalinity stress. - Chin. J. Plant Ecol. **27**: 491-498, 2016. [In Chin., ab: E]
- Panda, S.K., Yamamoto, Y., Kondo, H., Matsumoto, H.: Mitochondrial alterations related to programmed cell death in tobacco cells under aluminium stress. - Compt. rend. Biol. **331**: 597-610, 2008.
- Qiao, Z.J., Jing, T., Liu, Z.Q., Zhang, L.P., Jin, Z.P., Liu, D.M., Pei, Y.X.: H₂S acting as a downstream signaling molecule of SA regulates Cd tolerance in *Arabidopsis*. - Plant Soil **393**: 137-146, 2015.
- Qian, P., Sun, R., Ali, B., Gill, R.A., Xu, L., Zhou, W.J.: Effects of hydrogen sulfide on growth, antioxidative capacity, and ultrastructural changes in oilseed rape seedlings under aluminum toxicity. - J. Plant Growth Regul. **33**: 526-538, 2014.
- Shan, C., Liu, H., Zhao, L., Wang, X.: Effects of exogenous hydrogen sulfide on the redox states of ascorbate and glutathione in maize leaves under salt stress. - Biol. Plant. **58**: 169-173, 2014.
- Sheng, L.X., Feng, L.G., Shu, H.R.: Effects of nitrogen on the activity of antioxidant enzymes and functions of mitochondria in cherry rootstock roots under hypoxia stress. - Acta horticult. sin. **36**: 1575-1580, 2009 [In Chin., ab: E].
- Szabo, I., Zoratti, M.: Mitochondrial channels: ion fluxes and more. - Physiol. Rev. **94**: 519-608, 2014.
- Tian, B.H., Qiao, Z.J., Zhang, L.P., Hua, L., Pei, Y.X.: Hydrogen sulfide and proline cooperate to alleviate cadmium stress in foxtail millet seedlings. - Plant Physiol. Biochem. **109**: 293-299, 2016.
- Tonshin, A.A., Saprunova, V.B., Solodovnikova, I.M.: Functional activity and ultrastructure of mitochondrial isolated from myocardial apoptotic tissue. - Biochemistry **68**: 875-881, 2003.
- Wang, B.L., Shi, L., Li, Y.X., Zhang, W.H.: Boron toxicity is

- alleviated by hydrogen sulphide in cucumber (*Cucumis sativus* L.) seedlings. - *Planta* **231**: 1301-1309, 2010.
- Wang, J., Zhu, H., Liu, X., Liu, Z.: Oxidative stress and Ca^{2+} signals involved on cadmium-induced apoptosis in rat hepatocyte. - *Biol. Trace Element Res.* **161**: 180-189, 2014.
- Wang, Y.Q., Li, L., Cui, W.T., Xu, S., Shen, W.B., Wang, R.: Hydrogen sulfide enhances alfalfa (*Medicago sativa*) tolerance against salinity during seed germination by nitric oxide pathway. - *Plant Soil* **351**: 107-119, 2012.
- Xia, F.S., Wang, X.G., Li, M.L., Mao, P.S.: Mitochondrial structural and antioxidant system responses to aging in oat (*Avena sativa* L.) seeds with different moisture contents. - *Plant Physiol. Biochem.* **94**: 122-129, 2015.
- Yang, G.D., Zhao, K.X., Ju, Y.J., Mani, S., Cao, Q.H., Puukila, S., Khaper, N., Wu, L.Y., Wang, R.: Hydrogen sulfide protects against cellular senescence *via* S-sulphydration of Keap1 and activation of Nrf2. - *Antioxid Redox Signal.* **18**: 1906-1919, 2013.
- Yao, N., Eisfelder, B.J., Marvin, J., Greenberg, J.T.: The mitochondrion - an organelle commonly involved in programmed cell death in *Arabidopsis thaliana*. - *Plant J.* **40**: 596-610, 2004.
- Zhan, J., Li, W., He, H.Y., Li, C.Z., He, L.F.: Mitochondrial alterations during Al-induced PCD in peanut root tips. - *Plant Physiol. Biochem.* **75**: 105-113, 2014.
- Zhang, F., Wang, Y., Lou, Z., Dong, J.: Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorhiza*). - *Chemosphere* **67**: 44-50, 2007.
- Zhang, H., Hu, L.Y., Hu, K.D., He, Y.D., Wang, S.H., Luo, J.P.: Hydrogen sulfide promotes wheat seed germination and alleviates oxidative damage against copper stress. - *J. integr. Plant Biol.* **50**: 1518-1529, 2010b.
- Zhang, H., Jiao, H., Jiang, C.X., Wang, S.H., Wei, Z.J., Luo, J.P., Jones, R.L.: Hydrogen sulfide protects soybean seedlings against drought-induced oxidative stress. - *Acta Physiol. Plant.* **32**: 849-857, 2010a.
- Zhang, H., Ye, Y.K., Wang, S.H., Luo, J.P., Tang, J., Ma, D.F.: Hydrogen sulfide counteracts chlorophyll loss in sweet potato seedling leaves and alleviates oxidative damage against osmotic stress. - *Plant Growth Regul.* **58**: 243-250, 2009.
- Zhou, L., Wang N.N., Gong, S.Y., Lu, R., Li, Y., Li, X.B.: Overexpression of a cotton (*Gossypium hirsutum*) WRKY gene, *GhWRKY34*, in *Arabidopsis* enhances salt tolerance of the transgenic plants. - *Plant Physiol. Biochem.* **96**: 311-320, 2015.
- Zorov, D.B., Juhaszova, M., Sollott, S.J.: Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. - *Physiol. Rev.* **94**: 909-950, 2014.