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Research article

Hydrogen sulfide alleviates oxidative damage under excess nitrate stress through MAPK/NO signaling in cucumber

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A B S T R A C T

Hydrogen sulfide (H_2S) is emerging as a potential messenger molecule involved in modulation of physiological processes in plants. Mitogen-activated protein kinase (MAPK) and nitric oxide (NO) are essential for abiotic stress signaling. This work investigated the effects of H_2S and the crosstalk between H_2S, MAPK and NO in cucumber roots under nitrate stress. The inhibitory effect of 140 mM nitrate on the growth of shoot and root was substantially alleviated by treatment with H_2S donor sodium hydrosulfide (NaHS), especially 100 μM NaHS. Treatment with 100 μM NaHS reduced malondialdehyde (MDA) and H_2O_2 contents, ROS accumulation and increased the activities of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) and ascorbate peroxidase (APX). *CsNMAPK* transcript level was up-regulated by NaHS treatment, while significantly decreased by propargylglycine (PAG, specific inhibitor of H_2S biosynthesis) and hypotaurine (HT, H_2S scavenger) in cucumber roots under nitrate stress. NO accumulation was increased by NaHS treatment under nitrate stress, but reduced by HT, PAG and PD98059, indicating that NO might function downstream of MAPK and H_2S. MAPK inhibitor PD98059 and NO scavenger (cPTIO) reversed the alleviating effect of H_2S by increasing MDA and H_2O_2 contents, and decreasing antioxidant enzyme activities of SOD, CAT, POD, APX, and the endogenous H_2S contents and LCD activities under nitrate stress. In conclusion, H_2S played a protective role in cucumber seedlings under nitrate stress and MAPK/NO signaling were involved in the process by regulating antioxidant enzyme activities.

1. Introduction

For a long time, hydrogen sulfide (H_2S) has been thought as a product of cell metabolism, but nowadays is emerging as the third gaseous signaling molecule after nitric oxide (NO) and carbon monoxide (CO) in animals and plants. H_2S has been implicated in playing important roles in various development processes in plants. H_2S induced lateral root formation in tomato seedlings (Mei et al., 2017) and involved in stomatal movement (Garcia-Mata and Lamattina, 2010). Low concentrations of exogenous H_2S could improve seed germination and seedling growth of plants (Li et al., 2015; Zhou et al., 2018). However, high concentrations of H_2S are toxic to plants and inhibit their growth (Zhang et al., 2017). A lot of studies have reported that H_2S could alleviate various abiotic stress damage, such as salt (Wang et al., 2012), drought and osmotic stress (Chen et al., 2016a; Jin et al., 2011; Khan et al., 2017; Shan et al., 2011, 2018), cold stress (Du et al., 2017), high temperature (Chen et al., 2016b), and heavy metal (Chen et al., 2017; Rizwan et al., 2018). In plants, H_2S is predominantly produced by L-cysteine desulfhydrase (DES; EC 4.4.1.1) (Cheng et al., 2013).

Mitogen-activated protein kinase (MAPK) cascades are universal signaling modules in all eukaryotes and have been shown to play critical roles in many physiological and biochemical processes in plants (Song et al., 2015). MAPK cascades consist of three functionally associated protein kinases to activate the next one via phosphorylation. Overexpression of the maize mitogen-activated protein kinase gene (*ZmSIMK1*) increased tolerance to salt stress in *Arabidopsis* (Gu et al., 2010). In our previous study, we cloned a MAPK gene from cucumber and named *CsNMAPK*. Overexpression of *CsNMAPK* enhanced the nitrate stress tolerance of transgenic tobacco (Xu et al., 2013). In animals, H_2S-induced pancreatic acinar cell apoptosis is mediated via JNK and p38 MAP kinase (Adhikari and Bhatia, 2008). However, the mechanism of MAPK involved in H_2S-induced salt tolerance of plants remains elusive.

NO is another important second messenger, which plays roles in various plant physiological processes and in response to biotic and abiotic stresses (Jasid et al., 2008; Lobb et al., 2015; Qiao and Fan, 2008; Zhao et al., 2007; Zheng et al., 2009). Previous studies have confirmed that NO was involved in activating MAPK signaling pathways during the plant defense response (Lv et al., 2017). In plants, an

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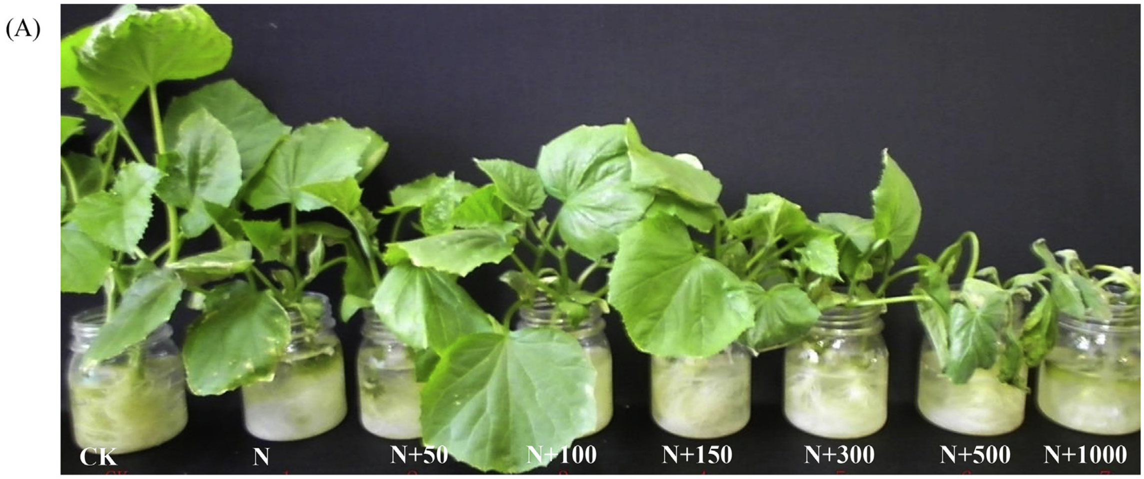

(B)

Treatment	Leaf weight(g)		Root weight(g)	
	Fresh weight	Dry weight	Fresh weight	Dry weight
CK	7.18±0.38a	0.53±0.02a	2.45±0.16a	0.08±0.00a
N	3.00±0.36c	0.36±0.02b	1.36±0.15bc	0.05±0.01cd
N+50	4.00±0.37bc	0.40±0.02b	1.61±0.08b	0.06±0.01abc
N+100	4.47±0.36b	0.42±0.04b	1.75±0.18b	0.08±0.01ab
N+150	3.37±0.44c	0.35±0.03bc	1.63±0.23b	0.06±0.01bcd
N+300	1.95±0.36d	0.27±0.03c	1.10±0.10c	0.05±0.00cd
N+500	1.60±0.20d	0.27±0.02c	0.95±0.07c	0.04±0.00d

Fig. 1. Effects of different concentration of NaHS on the growth of cucumber seedling under nitrate stress for 5 days. CK: control; N: 140 mM nitrate stress. 50, 100, 150, 300, 500 and 1000 μM NaHS were indicated. The phenotype (A), fresh weight and dry weight (B) of cucumber shoot and root after different treatment. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$).

interaction between H_2S and NO in modulating plant growth and development has been reported. H_2S enhanced salinity tolerance by NO pathway in alfalfa (Wang et al., 2012). H_2S may function downstream of NO (Li et al., 2013) or acts upstream of NO under abiotic stresses (Scuffi et al., 2014). In our previous study, we found nitrate reductase-dependent NO production was involved in H_2S-induced nitrate stress tolerance in tomato (Liang et al., 2018).

Cucumber is one of the most important vegetables in the world and widely grown in greenhouse of the north of China. Recently, over-utilization of chemical fertilizer has caused secondary salinization in Chinese greenhouse. The excessively accumulated anion and cation in soil of the greenhouse are NO_3^-, Ca^{2+}, and K^+ (Ju et al., 2007; Yang et al., 2010). Although the effects of H_2S on stress physiology have received much attention, little information was known about its effects on cucumber seedlings under nitrate stress. The interactions between H_2S, MAPK and NO in the acquisition of nitrate tolerance have not been reported. In this study, we investigated the function of H_2S and the involvement of MAPK and NO in alleviating the oxidative damage in root of cucumber seedlings.

2. Materials and methods

2.1. Plant materials and treatment

Cucumber (*Cucumis. sativus* L. cv. Jinyou 4) seeds were germinated on moisture filter paper in an incubator at 28 °C for 2 days. The germinated seeds were sown in sands. After 10 days, batches of 10 seedlings were transferred to plastic tank with 10 L nutrient solution with pH 6.0–6.5 containing aerated full nutrient solution according to Xu et al. (2008). The experiment was carried out under natural conditions in greenhouse of Kunming University of Science and Technology with the air temperature of 23–28 °C during the day and 13–18 °C during the night.

When seedlings were at three-leaf-stage, different treatments were given. These include: (1) normal nutrient solution (control); (2) normal nutrient solution + 140 mM nitrate (70 mM KNO_3 + 35 mM $Ca(NO_3)_2$); (3) normal nutrient solution + nitrate + 50, 100, 150, 300, 500, 1000 μM NaHS; (4) normal nutrient solution + 140 mM nitrate +100 μM NaHS; (5) normal nutrient solution + 140 mM nitrate + 100 μM NaHS + 100 μM MAPK inhibitor PD98059; (6) normal nutrient solution + 140 mM nitrate + 100 μM NaHS + 100 μM cPTIO. Samples of cucumber seedlings were taken after 0, 6 h, 1 d, 3 d, and 5 d of treatment and immediately frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Plant growth measurements

After 5 days of treatment, cucumber plants were sampled and divided into shoots and roots. Fresh weight (FW) of shoots and roots were determined directly. Then, the shoots and roots were dried at 80 °C for 48 h and weighed to determine the dry weight (DW).

2.3. Lipid peroxidation, H_2O_2 contents and antioxidant enzyme activities assay

Lipid peroxidation was determined by estimating the formation of malondialdehyde (MDA) using the thiobarbituric acid method described by Liang et al. (2018). H_2O_2 contents and antioxidant enzyme

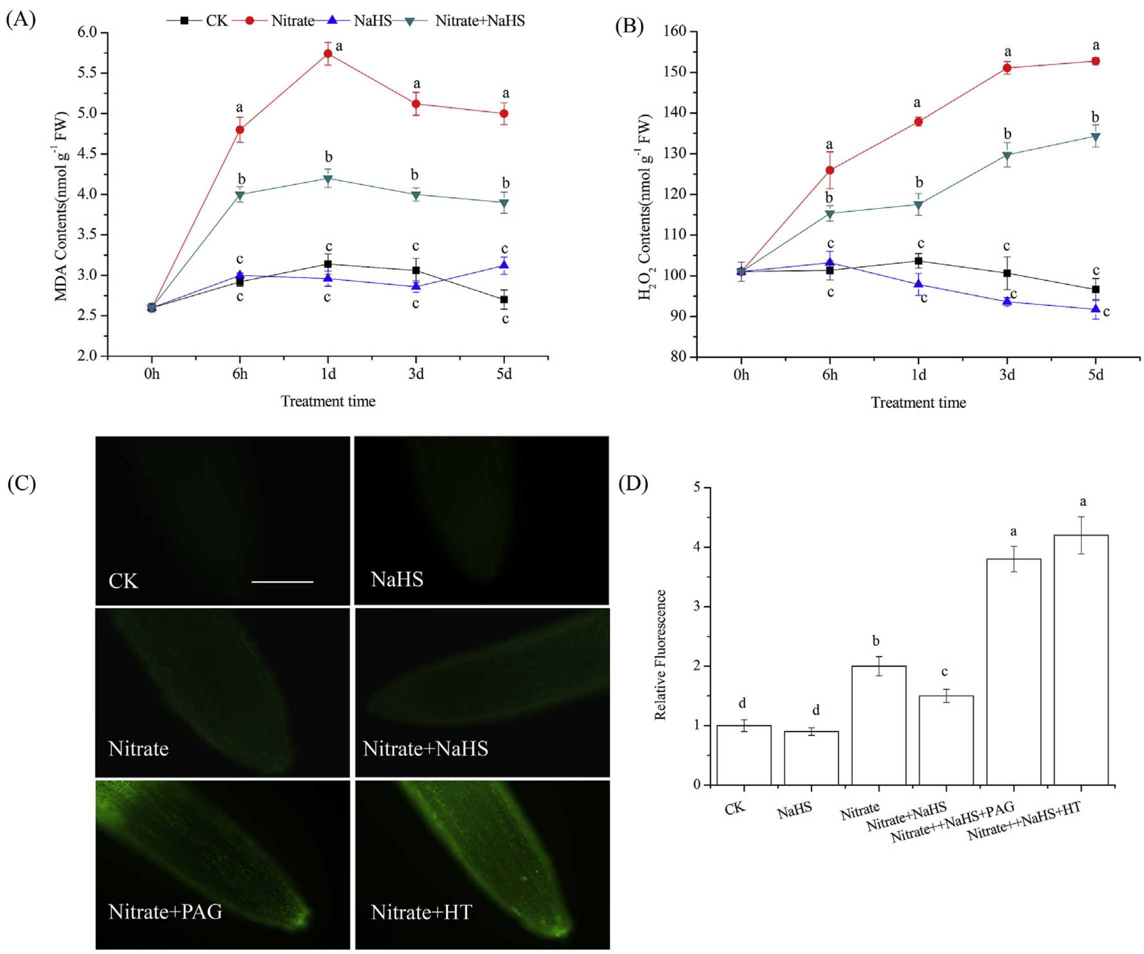

Fig. 2. Effect of NaHS on lipid peroxidation (A), H_2O_2 contents (B), and ROS accumulation (C) in cucumber root under nitrate stress. (D) ROS accumulation as relative fluorescence. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$). Scale bar = 100 μM.

activities of SOD, CAT, POD and APX were assayed according to the method as described by Liang et al. (2018).

2.4. Visualization of endogenous ROS and NO

The ROS accumulation was analyzed with 20 μM H_2DCFDA and the endogenous NO fluorescence was visualised using the fluorescent probe, 4-amino, 5-aminomethyl-2′,7′-difluorescein diacetate according to the method as described by Liang et al. (2018).

2.5. H_2S contents and L-cysteine desulfhydrase activities assay

The H_2S contents and L-cysteine desulfhydrase activities were analyzed according to the method of Li et al. (2012b) and Guo et al. (2018).

2.6. MAPK expression analysis by qRT-PCR

Total RNA was extracted using the TRIZOL reagent (Takara, Dalian, P. R. China) according to the manufacturer's instructions. Reverse transcription of RNA was carried out according to the instruction of the SYBR® PrimeScript™ RT-PCR Kit II (Takara, Dalian, China). Real-time quantitative PCR was performed using the iCycler iQ Real-time PCR detection system (Bio-Rad, Hercules, CA). To minimize sample variations, mRNA expression of the target gene was normalized relative to the expression of the house-keeping gene *Actin*. The primers used were: Actin (forward): 5′—CCACGAAACTACTTACAACTCCATC-3′; Actin (reverse): 5′-GGGCTG TGATTTCCTTGCTC-3′; MAPK (forward): 5′-AAGCGTTAGCACATCCGTACCT-3′; MAPK (reverse): 5′-CATCTCCTT CATCTGTTCTTCGTCT-3 ′. Three replicates were run for each sample.

2.7. Statistical analysis

All the experiments in this study were repeated at least three times of independent experiments, and the results shown were the mean ± SE of at least three independent experiments. Differences among treatments were analyzed by one-way ANOVA, taking $P < 0.05$ as significant according to Duncan's multiple range test.

3. Results

3.1. NaHS alleviates the growth of cucumber seedlings under nitrate stress

The effects of different concentrations of 50, 100, 150, 300, 500, 1000 μM NaHS on the growth of cucumber under nitrate stress were investigated. As shown in Fig. 1A, the cucumber seedling growth was inhibited by 140 mM nitrate treatment. When 50, 100, or 150 μM H_2S donor NaHS were added, the inhibition effect was alleviated, while 500 and 1000 μM NaHS aggravated the injury, indicating that a higher concentration of NaHS would be toxic (Fig. 1A). The fresh weight of shoot and root decreased by 58.3%, 45.1%, respectively, after 140 mM nitrate treatment for 5 days. NaHS supplementation significantly alleviated the reduction in leaf and root biomass, especially 100 μM NaHS (Fig. 1B). We then chose 100 μM NaHS to study the effect of exogenous

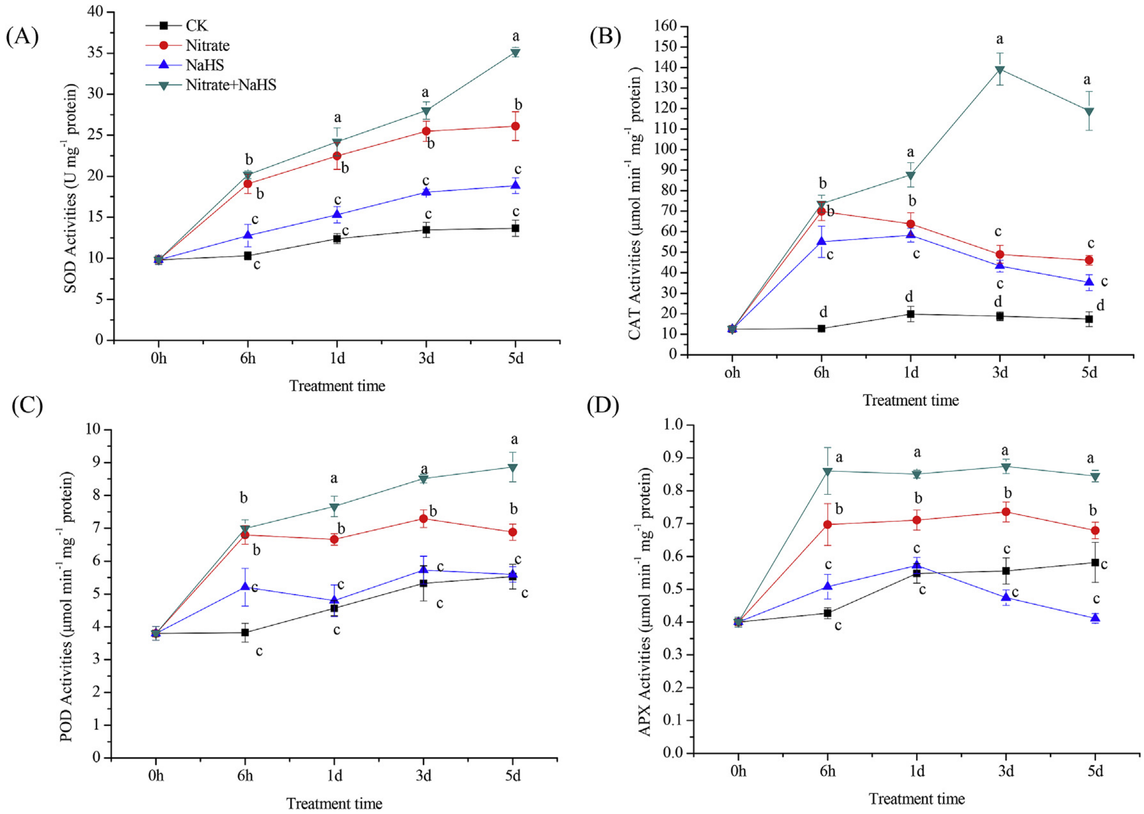

Fig. 3. Effect of NaHS on antioxidant enzyme activities of SOD (A), CAT (B), POD (C), and APX (D) in cucumber under nitrate stress. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$).

H_2S on the oxidative status of cucumber root under nitrate stress.

3.2. NaHS alleviates the oxidative damage caused by nitrate stress through enhancing antioxidant enzyme activities

Malondialdehyde (MDA) has been widely used to estimate the extent of lipid peroxidation in plant tissues. The MDA and H_2O_2 contents increased significantly after nitrate treatment for 6 h, 1, 3, 5 d, and NaHS addition dramatically decreased the MDA and H_2O_2 contents ($P < 0.05$). The MDA and H_2O_2 contents were decreased by 26.8% and 14.8%, respectively, after nitrate + NaHS treatment for 1 d, compared to the nitrate stress treatment (Fig. 2A and B).

Next, the ROS accumulation was analyzed with fluorescent probe H2DCFDA by epifluorescence microscope. As shown in Fig. 2C and D, the ROS accumulation increased after nitrate stress treatment and NaHS addition ameliorated this accumulation. HT and PAG treatment dramatically increased the ROS content, indicated that HT or PAG treatment brought about the aggravation of oxidative damage caused by nitrate exposure. Thus, these results indicated that the application of NaHS exhibited the protection against nitrate-induced oxidative damage in the root of cucumber.

To test the effects of NaHS treatment on the antioxidant systems of cucumber seedlings under nitrate stress conditions, we measured the ROS-scavenging enzyme activities of SOD, CAT, POD and APX. As shown in Fig. 3, there were no obvious changes of SOD, POD and APX activities in control and NaHS treatment during different treatment time. Nitrate stress significantly increased the activities of SOD, CAT, POD and APX from 6 h to 5 d, compared to the control ($P < 0.05$). After 1 d nitrate treatment, the activities of SOD, CAT, POD and APX increased by 65.1%, 341.5%, 46.1%, and 29.6%, respectively. Adding NaHS to the nitrate solution significantly increased the activities of SOD, CAT, POD and APX in cucumber roots from 1 to 5 d of treatment, compared to nitrate treatment. After 5 d treatment, the activities of SOD, CAT, POD and APX increased by 34.6%, 158.1%, 28.8%, and 24.4%, respectively, compared to nitrate stress.

3.3. MAPK expression and NO accumulation were increased by NaHS treatment under nitrate

MAPKs are important signaling molecules activated in response to a variety of environmental and developmental cues. Therefore, we studied whether *CsNMAPK* expression was involved in the process of H_2S alleviating nitrate stress. As shown in Fig. 4A, the *CsNMAPK* expression increased significantly after nitrate treatment. NaHS treatment also increased the expression of *CsNMAPK*. *CsNMAPK* expression under nitrate + NaHS treatment was 1.2- fold of the expression of nitrate stress treatment. H_2S inhibitor PAG and scavenger HT down regulated the *CsNMAPK* expression significantly ($P < 0.05$).

To evaluate if NO is involved in the process of H_2S alleviating nitrate stress, the NO accumulation in the root was analyzed used the NO-specific fluorescent probe, 4,5-diaminofluorescein diacetate (DAF-2DA). As shown in Fig. 4B and C, excess nitrate decreased the NO contents, while the NO contents were increased after NaHS added to the nitrate treatment, compared to the nitrate treatment alone. However, H_2S scavenger HT and H_2S inhibitor PAG decreased the NO content dramatically, indicating that NO and H_2S might have cross talk in the process of H_2S alleviating nitrate stress. Besides, MAPK inhibitor PD98059 also markedly inhibited the NO production, indicated that MAPK might function upstream of NO.

3.4. MAPK inhibitor and NO scavenger aggravated the oxidative damage caused by nitrate stress

To further investigate the role of MAPK and NO in the alleviating effect of NaHS, the cucumber seedlings were treated with MAPK inhibitor PD98059 and NO scavenger cPTIO. The MDA contents were

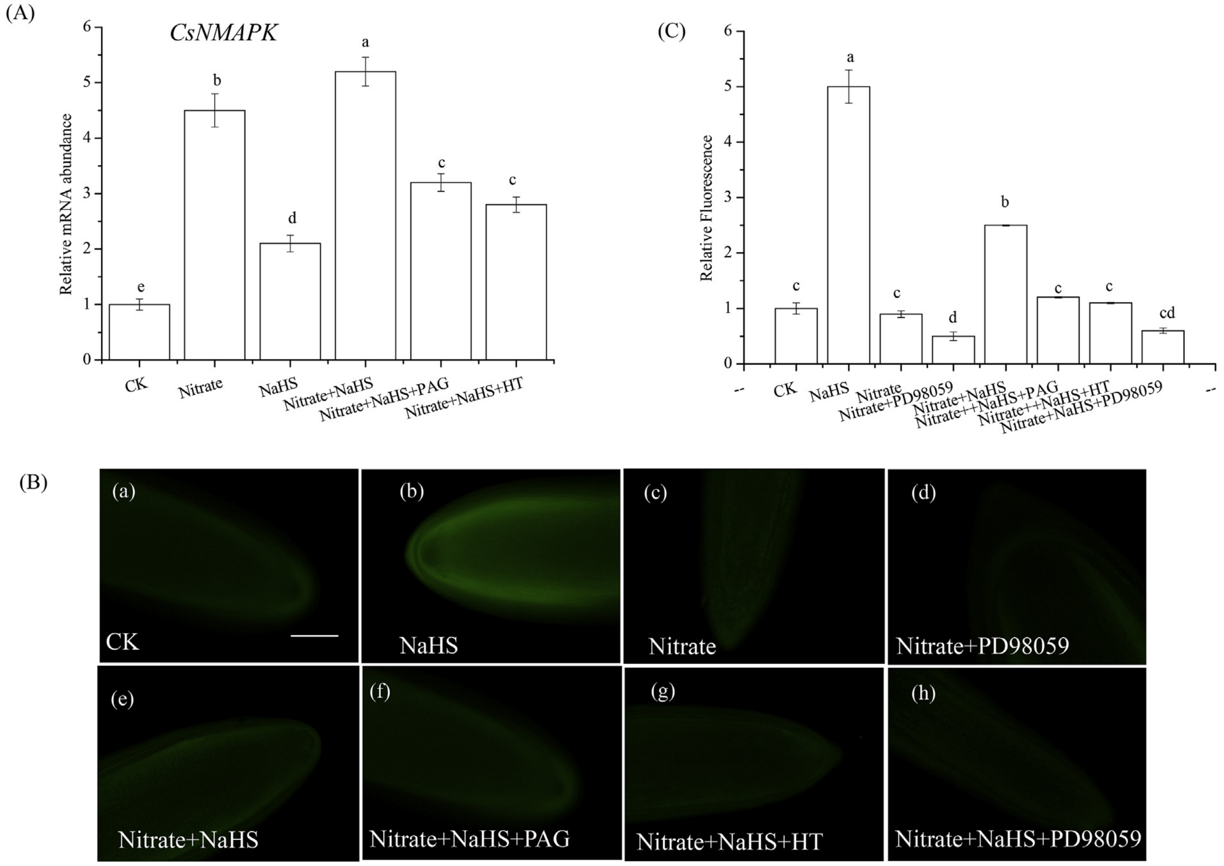

Fig. 4. Effect of NaHS on the expression of *CsNMAPK* and NO accumulation under nitrate stress. (A) *CsNMAPK* expression were analyzed by qRT-PCR in cucumber root under nitrate stress. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$). (B) Effect of NaHS and MAPK inhibitor PD98059 on the NO accumulation in cucumber root. (a): CK is control; (b): 100 μM NaHS treatment; (c): nitrate treatment; (d): NaHS and MAPK inhibitor PD98059 treatment; (e): nitrate + 100 μM NaHS treatment; (f): nitrate + NaHS + PAG treatment; (g): nitrate + NaHS + HT treatment; (h): nitrate + NaHS + PD98059 treatment. Scale bar = 100 μM. (C) NO production expressed as relative fluorescence.

increased significantly by 23.8% and 31.0%, respectively, after PD98059 and cPTIO added, compared with nitrate + NaHS treatment (Fig. 5A). The H_2O_2 contents were increased significantly by 23.8% and 9.4%, respectively, after PD98059 and cPTIO added, compared with nitrate + NaHS treatment ($P < 0.05$) (Fig. 5B). The results indicated that the alleviating oxidative damage caused by nitrate was reversed by PD98059 and cPTIO.

The effect of PD98059 and cPTIO on the activities of SOD, CAT, POD and APX were also investigated. As shown in Fig. 5C–F, the SOD, CAT, POD and APX activities were increased after NaHS added into the nitrate solution, compared to the nitrate treatment alone. When PD98059 and cPTIO were added, the SOD, CAT, POD and APX activities were decreased significantly ($P < 0.05$). The SOD, CAT, POD and APX activities decreased by 16.1%, 31.2%, 19.1%, 29.5% after PD98059 were added, and decreased by 12.0%, 30.4%, 20.1%, 31.8%, after cPTIO were added, respectively, compared to the nitrate + NaHS treatment.

3.5. *MAPK inhibitor and NO scavenger decreased the H_2S content and LCD activities caused by nitrate stress*

The effect of PD98059 and cPTIO on H_2S contents and LCD activities in cucumber root under nitrate stress were also investigated. As shown in Fig. 6A, exogenous application of NaHS resulted in significantly elevated H_2S contents and LCD activities in root compared with control plants ($P < 0.05$). Nitrate stress greatly decreased H_2S contents and LCD activities, while NaHS added plants were found to accumulate significantly higher H_2S contents and LCD activities in the roots compared with nitrate stress treatment plants. PD98059 treatment dramatically decreased the H_2S content and LCD activities by 19.6% and 11.8%, respectively, compared with nitrate + NaHS treatment. cPTIO treatment dramatically decreased the H_2S content and LCD activities by 45.1% and 33.7%, respectively, compared with nitrate + NaHS treatment. The present results clearly showed that MAPK and NO were required for the production of H_2S under nitrate stress.

4. Discussion

H_2S has been shown to act as signaling molecules in various physiological processes, playing significant roles in plant cellular processes, and also mediating responses to both biotic and abiotic stresses in plants (Khan et al., 2017). In our study, excess nitrate inhibited the growth of cucumber. However, low concentration of (50–150 μM) NaHS increased the root and shoot fresh and dry weight under nitrate stress, thus confirming previous observations that external application of NaHS significantly improved the physiological status of stressed plants (Chen et al., 2015; Deng et al., 2016).

ROS production is one of the major primary stress responses, if massive ROS production is not controlled, lipid membrane peroxidation can occur, resulting in oxidative damage at the cellular level. In cucumber roots, excess nitrate was observed to intensify ROS production and lipid peroxidation (Xu et al., 2012). In this study, the MDA, H_2O_2 and ROS accumulation were alleviated by the addition of NaHS (Fig. 2). H_2S might act as an antioxidant to inhibit or scavenge ROS productions for maintaining the lower MDA and H_2O_2 levels, and thereby preventing oxidative damages (Chen et al., 2017). The protective

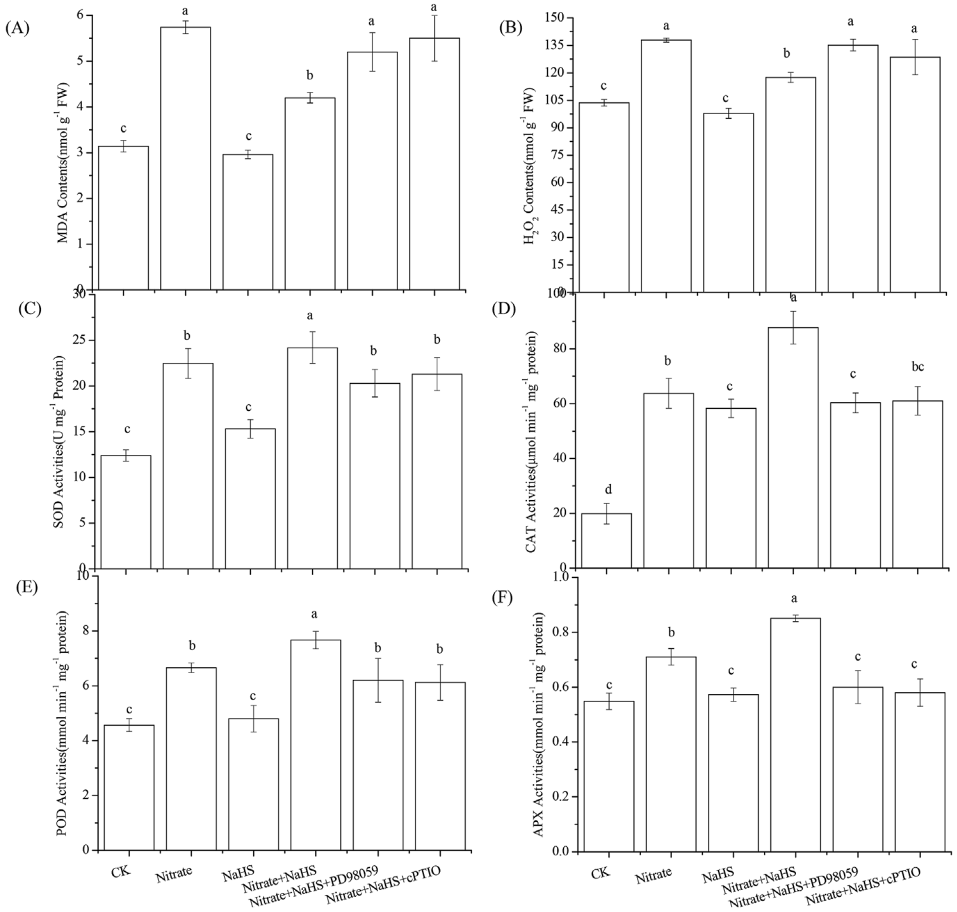

Fig. 5. Effect of MAPK inhibitor PD98059 and NO scavenger cPTIO on the lipid peroxidation (A), H_2O_2 (B) contents and antioxidant enzyme activities of SOD (C), CAT (D), POD (E), and APX (F) in cucumber root under NaHS and nitrate treatment. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$).

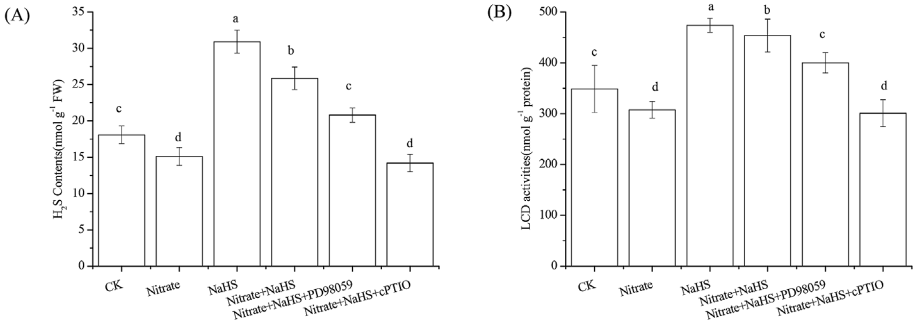

Fig. 6. Effect of MAPK inhibitor PD98059 and NO scavenger cPTIO on the H_2S content (A) and L-cysteine desulfhydrase activities (B) in cucumber root under NaHS and nitrate stress treatment. Data are mean values ± SD of three independent experiments. Error bars with different letters indicate significant differences ($p < 0.05$).

mechanism of H_2S against oxidative stress was correlated with the enhanced activities of ROS- and detoxifying enzymes under abiotic stress (Mostofa et al., 2015). NaHS treatment alleviated the ROS burst and cell damage induced by abiotic stress, via modulating metabolisms of CAT, POD and GR in bermudagrass (Shi et al., 2013). Pretreatment with H_2S donor NaHS enhanced heat tolerance in relation to antioxidant system in maize seedlings (Li et al., 2014). In our study, higher activities of SOD, CAT, POD and APX in NaHS treated root under nitrate stress were likely to be responsible for the elimination of ROS and the reduction of oxidative damage caused by nitrate treatment (Fig. 3).

MAPK cascades are ubiquitous signaling modules in eukaryotes. In animals, hydrogen sulfide induced apoptosis of human aorta smooth muscle cells via the activation of MAPKs (Yang et al., 2004). Exogenous hydrogen sulfide exhibits anti-cancer effects though p38 MAPK signaling pathway in C6 glioma cells (Zhao et al., 2015). In plants, H_2S alleviates the cold stress through MAPK4 in *Arabidopsis* (Du et al., 2017). In our study, *CsNMAPK* expression was increased by NaHS and decreased by NaHS inhibitor and scavenger (Fig. 4A), indicating that the MAPK expression were modulated by NaHS treatment. The decreased MDA and H_2O_2 contents and enhanced antioxidant enzyme activities by NaHS treatment were reversed by MAPK inhibitor PD98059, indicating that the activation of MAPK was required for the alleviating effect of NaHS.

Previous studies have revealed an interesting relationship between NO and MAPK activation in plants in response to various stresses or stimuli, the crosstalk between MAPK and NO during NaHS treatment, however, is not yet clear. NO was involved in activating MAPK signaling pathways during the plant defense response (Clarke et al., 2000; Lv et al., 2017). In tomato, acclimation-induced NO accumulation played a role in cold response by activating MPK1/2 (Lv et al., 2017).

MAPK cascades are also important regulators for NO production. MAPK6 in *Arabidopsis*, can phosphorylate nitrate reductase 2, which in turn increases NR activity and NO production in response to H_2O_2 during *Arabidopsis* root development (Wang et al., 2010). In our study, the MAPK inhibitor decreased the NO accumulation, indicating that MAPK might function upstream of NO (Fig. 4B).

More and more research indicated that there existed a cross-talk between H_2S and NO responsible for the increased abiotic stress tolerance. NO was involved in the NaHS-induced alleviation of abiotic stress, such as cadmium toxicity in alfalfa (Li et al., 2012a) and bermudagrass seedlings (Shi et al., 2014), salt tolerance in barley seedling roots (Chen et al., 2015). Exogenous H_2S-induced NO production was mediated by ROS and the modulation required the activation of MAPK6 (Zhang et al., 2017). In this study, we observed that the NO fluorescence increased after nitrate + NaHS treatment, but were decreased by HT and PAG, indicating that NO was involved in the nitrate stress tolerance inducing by H_2S in cucumber roots. NO-induced synthesis of hydrogen sulfide alleviated osmotic stress in wheat seedlings through sustaining antioxidant enzymes, osmolyte accumulation and cysteine homeostasis (Khan et al., 2017). In our study, NO scavenger reverse the alleviating effect of H_2S by regulating antioxidant enzyme activities.

5. Conclusion

In conclusion, proper concentration of H_2S could protect cucumber seedlings alleviating the oxidative damage caused by excess nitrate stress by modulating the antioxidant enzyme activities. MAPK and NO were involved in the function of H_2S under nitrate stress and MAPK might be upstream of NO signaling.

Conflicts of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Qi Qi: Formal analysis. **Zhaolai Guo:** Formal analysis. **Yuanlin Liang:** Formal analysis. **Huini Xu:** Formal analysis.

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