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# Effects of Hydrogen Sulfide on Cold-Induced Oxidative Damage in *Cucumis sativus* L.

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**Abstract**

One of the major abiotic stresses limiting the productivity and the geographical distribution of many important crops is low temperature. Hydrogen sulfide ($H_2S$) is an important signaling molecule involved in several stress-resistance processes such as drought, salinity and heavy metal stresses in plants. The aim of this study was to investigate the effects of exogenous $H_2S$ on improving chilling tolerance of cucumber seedlings. The results indicated that seedlings exposed to chilling stress (4 °C) increased the level of electrolyte leakage, lipid peroxidation, hydrogen peroxide, proline content and guaiacol peroxidase (GPX) activity; while sugar soluble content decreased. Pretreatment with sodium hydrosulfide (NaHS), a hydrogen sulfide donor, slightly reduced the malondialdehyde content, hydrogen peroxide content and electrolyte leakage, which were induced by chilling stress and also elevated the activity of antioxidant enzymes, soluble sugar and proline levels, and reduced glutathione content in plants under chilling stress condition. Pre-treatment with other $Na^+$ and sulfur-containing components including $Na_2S$, $Na_2SO_4$, $Na_2SO_3$ showed no significant effect on lipid peroxidation and hydrogen peroxide content under chilling stress. It can be concluded that the effect of NaHS pretreatment on alleviation of cold stress damages is probably related to its ability to release $H_2S$ because $Na^+$- or sulfur-containing compounds (except NaHS) had no similar effects on alleviation of chilling damages.

**Keywords**: Antioxidant enzymes, *Cucumis sativus* L, Hydrogen sulfide, Lipid peroxidation, Proline.

**Abbreviation:** ROS: Reactive oxygen species; SOD: Superoxide dismutase; CAT: Catalase; POD: Peroxidase; APX: Ascorbate peroxidase; $H_2S$: Hydrogen sulfide; NaHS: Sodium hydrosulfide; MDA: Malondialdehyde; TCA: Trichloroacetic acid; GSH: Reduced glutathione.



## Introduction

Low temperatures (0–15ºC), but not freezing temperatures, can damage many plant species (Zhu et al., 2007). As a result of exposure to low temperatures, many physiological and biochemical cell functions disturb, resulting in visible adverse symptoms such as wilting, chlorosis, or necrosis (Ruelland and Zachowski, 2009) as well as ultra-

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structural changes in a wide range of cell components, including plastids, thylakoid membranes and the phosphorylation of thylakoid proteins, and mitochondria (Zhang et al., 2011).

Reactive oxygen species (ROS) are usually increased in plants exposed to low temperatures. ROS produced as a result of low temperature exposure are highly reactive and can severely impair the normal functions of plant cells by lipid peroxidation, protein degradation, DNA fragmentation, and may eventually cause cell death (Sun et al., 2010). To keep ROS in low levels, plants have anti-oxidative systems composed of enzymatic scavengers, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX) and glutathione reductase (GR), and also some non-enzymatic antioxidants, such as ascorbic acid, α-tocopherols, phenolic compounds and reduced glutathione (Garbero et al., 2011). Many studies have demonstrated that antioxidant systems play important roles in protecting plants against oxidative damage induced by cold stress. The regulation of these antioxidants by an exogenous substance might mediate the activity of antioxidant enzymes in plant to improve their cold tolerance (Liu et al., 2011). For instance, it has been shown that application of growth stimulants such as aminochelates can significantly improve the growth of pepper seedlings under cool temperature (Souri and Yaghoubi, 2019).

A number of studies have shown that hydrogen sulfide ($H_2S$) acts as an important signaling molecule to regulate many physiological processes in mammalian cells (Wang, 2002; Lefer, 2007). Although the information on the role of $H_2S$ in mediating plant physiological metabolism is limited, a few research groups have paid more attention to the *in vivo* role of $H_2S$ in plant development and stress response in recent years. For example, Hydrogen sulfide has been shown to promote root organogenesis in *Ipomoea batatas* L., *Salix matsudana* 'Tortuosa' and *Glycine max* L. (Zhang et al., 2009). $H_2S$ can alleviate toxicity induced by heavy metal stresses such as cadmium stresses (Sun et al., 2013) in different plant species (Zhang et al., 2010a). Pretreatment with $H_2S$ donors (e.g. sodium hydrosulfide, NaHS) confers improvement of heat tolerance in tobacco and maize (Li et al., 2012b; Li et al., 2013a), enhanced drought tolerance via interacting with abscisic acid (ABA) in stomatal regulation in Arabidopsis (Jin et al., 2013), and improved tolerance to salt and osmotic stresses via modification of reactive oxygen species biosynthesis in strawberry plants (Christou et al., 2013). Additionally, $H_2S$ enhances photosynthesis in *Spinacia oleracea* L. seedlings through promoting chloroplast biogenesis, photosynthetic enzyme expression, and thiol redox modification (Chen et al., 2011). However, this is just the beginning for the study of $H_2S$ mediated plant development and stress response, and the exact physiological and molecular mechanisms remain unclear. Little is known about the roles of $H_2S$ in responses to chilling stress. In many places with arid and semi-arid climates, cucumber is cultivated in the winter with high probability of low temperature damages; therefore the application of exogenous substrate that can alleviate the stress damage is of vital importance. Therefore, the aim of this study was to investigate the effects of $H_2S$ pretreatment on alleviation of cold stress damages in cucumber plant.

## Materials and methods

Cucumber (*Cucumis sativus* L.) seeds were placed on moist gauze at 25 °C for 2 d to germinate and then planted into plastic pots filled with perlite. The seedlings were irrigated with half-strength of Hoagland solution once per day for four weeks. After four weeks of growth, leaves of plants were sprayed with 10, 20, 40 or 80 µM NaHs ($H_2S$ donor) solutions for five days.

Control plants were also sprayed with distilled water. On the sixth day, all of plants were divided into two groups, one group of plants were exposed to 4°C for 3 h in refrigerator and other group was in control condition (room temperature). Three days after chilling stress, electrolyte leakage of plants was measured. According to the results of this experiment, the optimal NaHS concentration that decrease the electrolyte leakage in cucumber seedlings during chilling stress was determined (40 and 80 µM). In the second part of the experiment, $Na^+$ and sulfur-containing components, such as $Na_2S$, $Na_2SO_4$ and $Na_2SO_3$ were also used as control of the $H_2S$ donor. In this step, after four weeks of growth, plants were sprayed with only 40 or 80 µM NaHs for five days. These rates were optimized based on the results of the previous experiment. Control plants were also sprayed with distilled water. On the sixth day, half of the plants were exposed to 4°C for 3 h for 3 d and the other half were transferred to the greenhouse (25°C). After 24 h of chilling stress the 3rd leaf (in acropetal order) of each plant was harvested and immediately frozen in liquid nitrogen and stored at -80 °C for future analysis.

***Determination of electrolyte leakage***

Electrolyte leakage was estimated using the method of Ben Hamed et al. (2007). Leaf samples were cut into small 5 mm sections and put in test tubes containing 8 ml deionized distilled water. The tubes were placed in a water bath at 32 °C for 2 h. Initial electrical conductivity of the medium ($EC_1$) was assessed. The samples were then placed in an autoclave at 121 °C for 20 min to expel all electrolytes. Samples were cooled at 25 °C and second electrical conductivity ($EC_2$) was measured. Total electrolyte leakage was calculated by using the following formula:

$$\text{Electrolyte leakage } (\%) = \frac{Ec_1}{Ec_2} \times 100$$

***Lipid peroxidation***

The level of lipid peroxidation, as an indicator of cellular damage, was measured in terms of malondialdehyde (MDA) content according to Heath and Packer (1968). Leaf samples (0.1 g) were homogenized in 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 4000×g for 10 min. The supernatant (0.5 ml) was mixed with 1.5 ml of 20% (w/v) TCA containing 0.5% (w/v) 2-thiobarbituricacid (TBA). Mixtures were heated at 95 °C for 30 min then quickly cooled in an ice bath. Mixtures were centrifuged at 10000×g for 5 min and their supernatant absorbance was measured at 532 nm. The value of non-specific absorption at 600 nm was subtracted from the 532 nm reading. The MDA content was calculated using the Lambert-Beer law, with extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as µmol MDA per g fresh weight.

***Hydrogen peroxide content***

Hydrogen peroxide ($H_2O_2$) levels were determined according to Alexieva et al. (2001). Fresh leaves (0.5 g) were homogenized in ice bath with 5 ml 0.1% (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 4000×g for 15 min and 0.5 mL of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M KI. The reaction was carried out for 1 h in darkness and absorbance was measured at 390 nm. The amount of hydrogen peroxide was calculated using a standard curve prepared with known concentration of $H_2O_2$.

***Total soluble sugar and Proline determination***

Total soluble sugar was determined using anthrone reagent and glucose as standard (Roe, 1955). Determination of free proline content performed according to Bates et al. (1973).

***Measurement of reduced glutathione (GSH)***

Reduced GSH was estimated following the modified method of Ellman (1959). About

200 mg of the leaves were homogenized in 4 mL of 15% meta-phosphoric acid (W/V) and centrifuged at 10000×g for 30 min at 48 °C. Aliquots of 0.2 mL of the supernatant were mixed with 2.6 mL 150 mM K-phosphate buffer (pH 7.7) and 0.2 mL 5,5-Dithio-bis (2-Nitrobenzoic acid) (DTNB). The color was allowed to develop for 30 min at room temperature, and the absorbance of the clear supernatant was recorded at 412 nm. Reduced glutathione was calculated using a standard curve prepared with known concentration of GSH.

***Enzyme extraction and activity determination***

Leaves (500 mg) were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1% soluble PVP and 1 mM EDTA. The homogenate was centrifuged at 20000×g for 20 min and the supernatant used for assay of the activity of enzymes.

***Catalase (CAT) activity (EC 1.11.1.6)***

Catalase activity was assayed by measuring the initial rate of $H_2O_2$ disappearance at 240 nm using the extinction coefficient of 40 $mM^{-1}$ $cm^{-1}$ for $H_2O_2$ (Dhindsa et al., 1981).

***Guaiacol peroxidase (GPX) activity (EC1.11.1.7)***

The GPX activity was determined using the method of Plewa et al. (1999) following the formation of tetraguaiacol by measuring the absorbance at 470 nm and using an extinction coefficient of 25.5 $mM^{-1}$ $cm^{-1}$.

***Ascorbate peroxidase (APX) activity (EC 1.11.1.11)***

Ascorbate peroxidase was determined spectrophotometrically according to the oxidation of ASA. The reaction solution contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbate, 0.1 mM $H_2O_2$ and 150 µL enzyme extract. $H_2O_2$-dependent oxidation of ASA was followed by measuring the decrease in absorbance within 1 min at 290 (extinction coefficient of 2.8 $mM^{-1}$ $cm^{-1}$) (Nakano and Asada., 1981).

***Total soluble proteins***

Protein content was determined according to the method of Bradford (1976) using bovine serum albumin as standard.

***Statistical analysis***

The experiments were performed in factorial experiment based on completely randomized design with three replications. Statistical analyses were conducted by SAS software (version 9.3. SAS Institute, Inc., Cary, NC, USA 2011) using two factorial ANOVA to present interactions between the two factors (temperature and hydrogen sulfide). The significant difference between treatments was evaluated using the Duncan's test at $P \leq 0.05$.

## Results

***Electrolyte leakage***

Electrolyte leakage is one of the membrane injury indices, which has been used to determine the negative impact of stress on cellular stability. The results showed that electrolyte leakage increased by chilling stress (about 5 fold). Meanwhile, application of $H_2S$ under chilling stress significantly decreased (about 50%) the electrolyte leakage in cucumber plants (Fig. 1). $H_2S$ pretreatment had no significant effect on plants under control condition.

***Lipid peroxidation***

Malondeladehyde and other aldehydes were measured as an indicator of lipid peroxidation. The data showed that chilling stress damaged the membrane and increased the amount of MDA and other aldehydes contents (Fig. 2A and B). Pre-treatment of plants with 40 and 80 µM NaHS decreased the amounts of MDA and other aldehydes (82-85%) in plants exposed to cold stress condition. However, pretreatment of plants with other sulfide salts had no significant effects on the level of lipid peroxidation in plants exposed to chilling stress condition (Table 1).

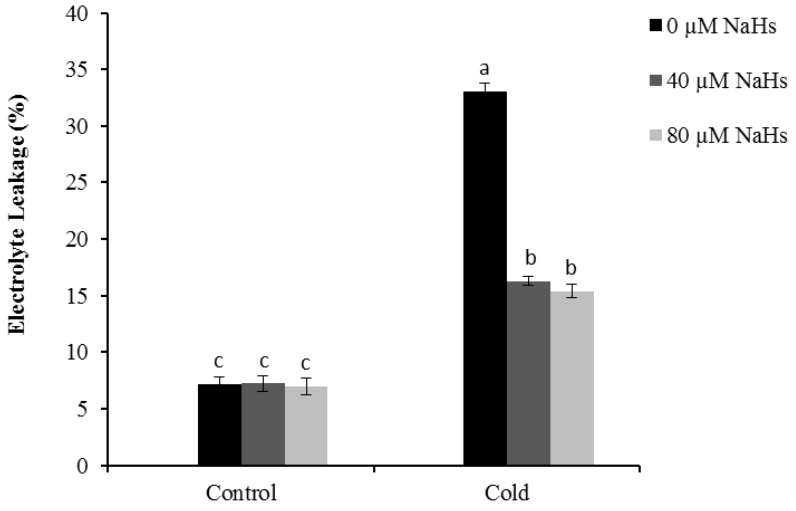


**Fig. 1. Effect of different concentrations of NaHS pretreatment on electrolyte leakage in cucumber plant under control and cold stress conditions. Means with the same letters are not significantly different according to Duncan's test at P<0.05 significant level**

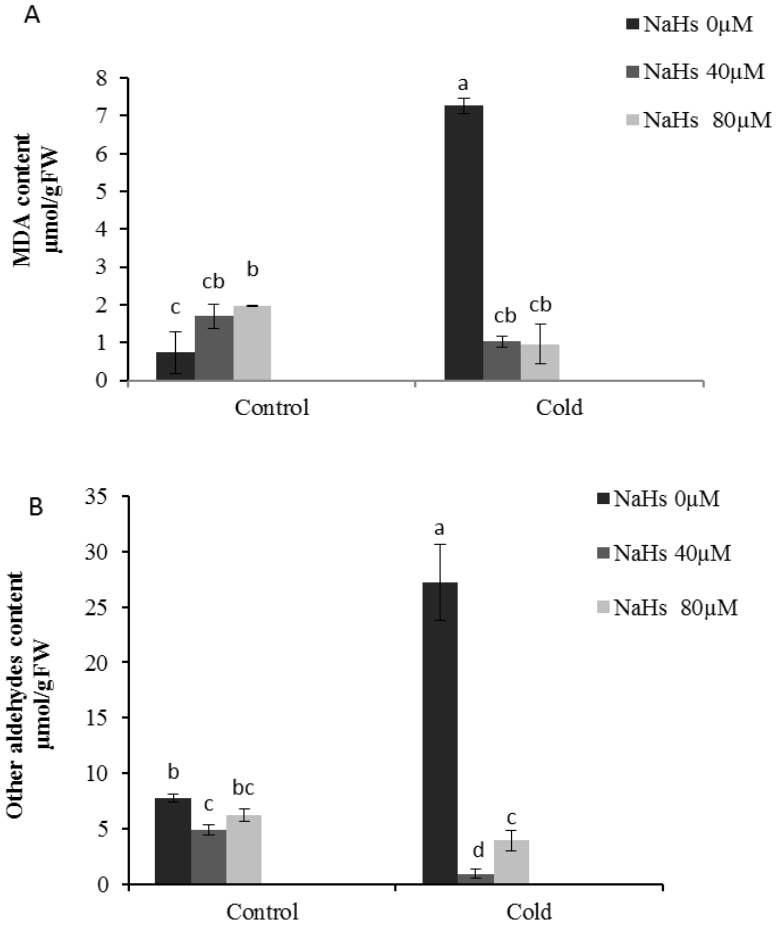


**Fig. 2. Effect of different concentrations of NaHS pretreatment on Malondeladehyde (MDA, A) and other aldehydes (B) content in cucumber plant exposed to control and cold stress conditions. Means with the same letters are not significantly different according Duncan's test at P<0.05 significant level**

**Table 1. Effect of NaHs, $Na_2S$, $Na_2SO_3$ and $Na_2SO_4$ pretreatments on Malondeladehyde (MDA), other aldehydes and hydrogen peroxide content in cucumber plant under control and chilling stress conditions. The mean comparisons of treatments were done using Duncan's test at P<0.05 significant level**

| Treatment | MDA | Other aldehyde | $H_2O_2$ |
|---|---|---|---|
| NaHS ( 0µM) +Control | 0.73±0.56 | 7.76±0.31 | 322.15±42.49 |
| NaHS ( 0µM)+Cold | 7.26±0.20 | 27.2±3.43 | 429.85±26.64 |
| NaHS (40µM)+Control | 1.7±0.32 | 4.88±0.44 | 243.69±26.20 |
| NaHS (40µM)+Cold | 1.03±0.14 | 0.94±0.40 | 300.1±51.96 |
| $Na_2S$ (40µM)+Control | 2.07±0.68 | 8.68±0.69 | 261.64±9.07 |
| $Na_2S$ (40µM)+Cold | 6.06±0.80 | 24.65±3.91 | 384.21±7.76 |
| $Na_2SO_3$ (40µM)+Control | 1.08±0.43 | 11.65±0.99 | 249.85±3.20 |
| $Na_2SO_3$ (40µM)+Cold | 4.6±0.37 | 24.72±1.55 | 409.33±16.11 |
| $Na_2SO_4$ (40µM)+Control | 2.4±0.13 | 14.43±1.37 | 249.33±11.59 |
| $Na_2SO_4$ (40µM) +Cold | 6.45±1.28 | 27.71±3.37 | 392.41±6.42 |

### *Hydrogen peroxide content*

As shown in the Fig 3, chilling stress caused an increase in $H_2O_2$ content, which is indicative of stress induced oxidative stress in plants. In plants under chilling stress, hydrogen sulfide (40 and 80 µM) pretreatment significantly decreased the amount of $H_2O_2$ (30-35% respectively). However in control plants, $H_2S$ had no significant effect on $H_2O_2$ content. Results also showed that in comparison with NaHS, other sulfide salts had no significant effect on the reduction of $H_2O_2$ and alleviation of chilling stress (Table 1).

### *Total soluble sugar*

Hydrogen sulfide pretreatment increased total soluble sugar (100%) in stress-exposed plants in comparison with non-pretreated plants. Application of $H_2S$ had no significant effect in total soluble sugar in control condition (Fig. 4).

### *Proline content*

Measurement of proline as one of the main osmo-protectant in plant showed that proline content increased about 5 fold in cucumber plants that were exposed to cold stress (Fig. 5). In control plants, pretreatment of plants with $H_2S$ increased the proline content about 4-5 fold. In control plants the effect of 40 µM $H_2S$ was more than the effect of 80 µM on proline content; however in plants that were exposed to cold stress, $H_2S$ pretreatment increased proline content about 60%. In cold exposed plants, the effects of 80 µM $H_2S$ on proline content was more than the effects of 40 µM.

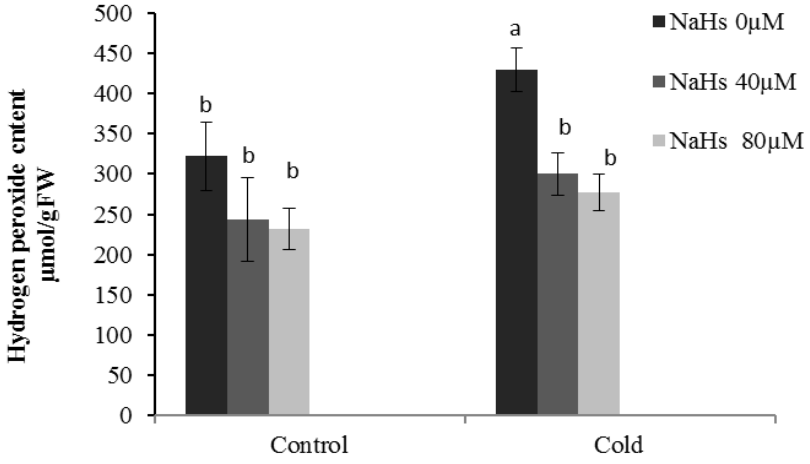


**Fig. 3. Effect of different concentrations of NaHS pretreatment on hydrogen peroxide content in cucumber plant under control and chilling stress conditions. Means with the same letters are not significantly different according to Duncan's test at P<0.05 significant level**

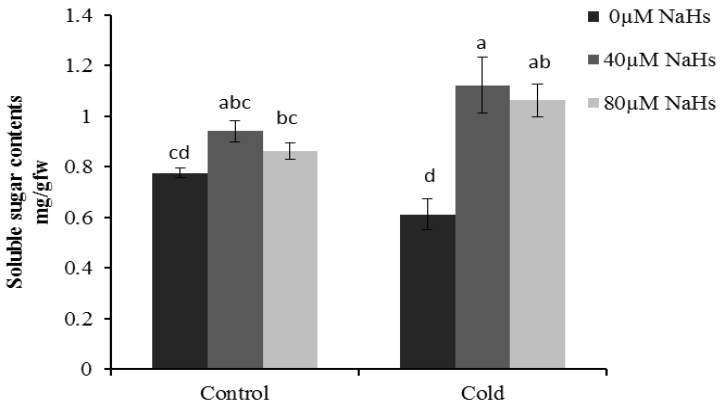


**Fig. 4. Effect of different concentrations of NaHS pretreatment on soluble sugar in cucumber plant under control and chilling stress conditions. Means with the same letters are not significantly different according to Duncan's test at P<0.05 significant level**

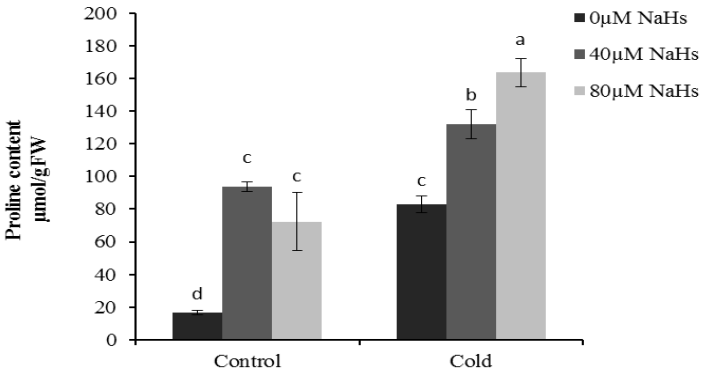


**Fig. 5. Effect of different concentrations of NaHS pretreatment on proline content in cucumber plant under control and chilling stress conditions. Means with the same letters are not significantly different according to Duncan's test at P<0.05 significant level**

### *Reduced glutathione content*

As it is shown in Fig 6, chilling stress had no significant effects on GSH content, while GSH concentration doubled in cold exposed plants that were pretreated with 40 µM $H_2S$. Pretreatment of plants with 80 µM $H_2S$ increased the GSH in about 68% under cold stress. Application of $H_2S$ did not result in any significant effects on GSH under control condition.

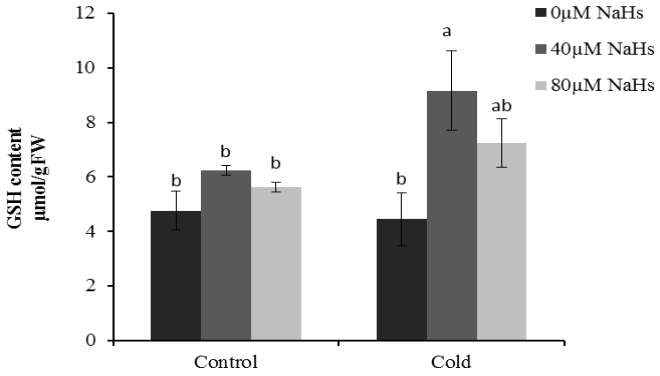


**Fig. 6. Effect of different concentrations of NaHS pretreatment on reduced glutathione content in cucumber plant under control and chilling stress conditions. Means with the same letters are not significantly different according to Duncan's test at P<0.05 significant level**

***Antioxidant enzyme activities***

In the present research, the effect of chilling stress on CAT, GPX and APX in cucumber plant leaves, either with or without $H_2S$ pretreatment was studied. As shown in Fig 7-A, B and C, the activity of CAT and APX was not changed in stressed plants compared with their activity in control groups. However, GPX activity increased in plants exposed to cold stress. Application of $H_2S$ pretreatment increased the activity of CAT and GPX (45 and 100% respectively) compared to their activity in non-pretreated plants. Only pretreated plants with 80 µM $H_2S$ significantly increased the APX activity under cold stress condition (approximately 62%).

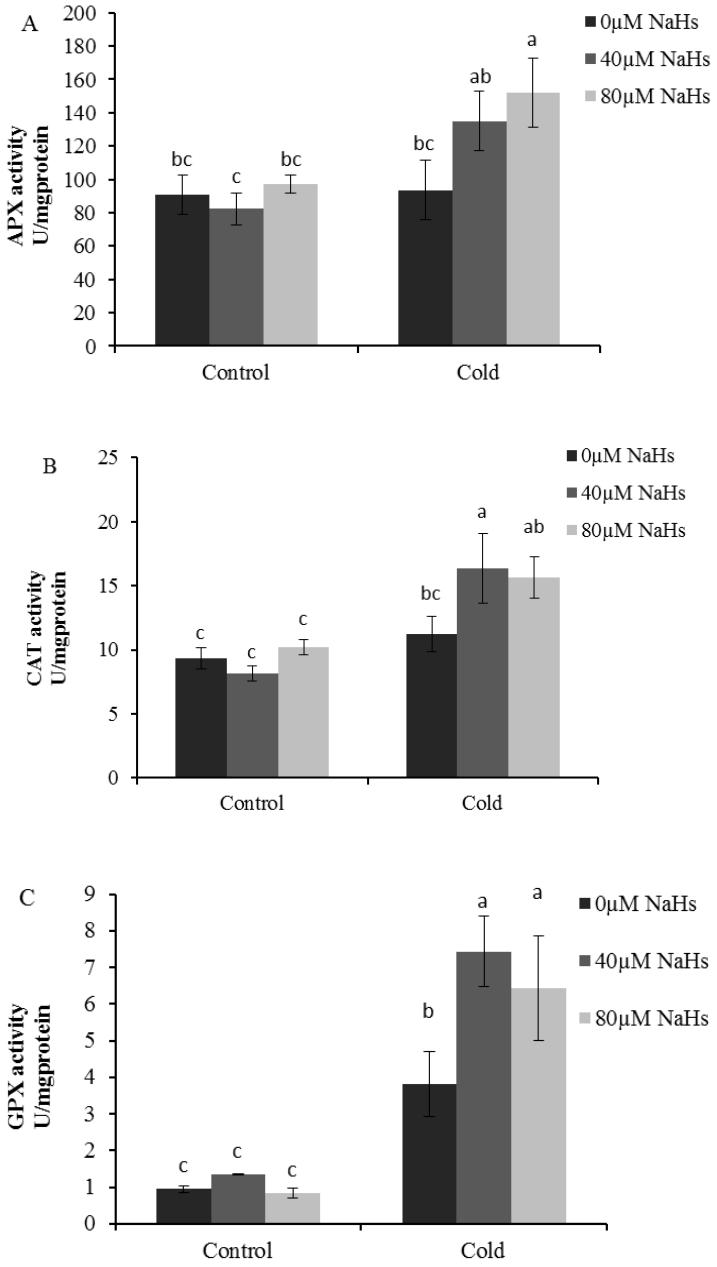


**Fig. 7. Effect of different concentrations of NaHS pretreatment on activity of ascorbate peroxidase (APX, A), catalase (CAT, B), and glutathione peroxidase (GPX, C) in cucumber plant under control and chilling stress conditions. Means with the same letters are not significantly different according to Duncan's test at P<0.05 significant level**

## Discussion

### *$H_2S$ pretreatment decreases electrolyte leakage and lipid peroxidation*

Membrane functional and structural stability is crucial for plant adaptation to cold and other stresses (Bharwana et al., 2014). Membranes are the primary sites for cold-induced damages. Changes in the structure of membranes from flexible liquid-crystal into solid gel structure alter membrane properties and membrane-bound enzyme activity (Hana and Bischofa, 2004). Many previous studies have shown that $H_2S$ application reduces the permeability of plasma membrane and significantly improves the growth of the plants (Shi et al., 2013; Bharwana et al., 2014). Fu et al. (2013) reported that *Vitis vinifera* plants treated with NaHS have improved chilling resistance through decrease of membrane permeability (Fu et al., 2013). Recently Janicka et al. (2017) demonstrated that nitric oxide, $H_2O_2$ and $H_2S$ as signaling molecules influence plasma membrane $H^+$-ATPase (EC 3.6.3.14) activity in conditions of both salt (50 mM NaCl) and low temperature (10°C, LT) stresses and alleviate the damage induced by stresses (Janicka et al., 2017). Therefore, it seems that $H_2S$ can protect membrane stability through modification of $H^+$-ATPase activity and reduction of lipid peroxidation. It has been previously reported that membrane integrity decreases as a result of exposure to cold stress through peroxidation of membrane lipids (Kuk et al., 2003), and MDA, a common product of lipid peroxidation, reflects the extent of oxidative injury (Tambussi et al., 2004). In this experiment MDA and other aldehyde contents increased in plants exposed to cold stress and $H_2S$ pretreatment decreased lipid peroxidation level. The results are in consistent with those of Fu et al. (2013) and Ali et al. (2014), who indicated that protective role of $H_2S$ at low concentrations was through reaction with lipid radicals and inhibition of lipid peroxidation (Fu et al., 2013; Ali et al., 2014). It has also been shown that NaHS dissociates to $Na^+$ and $HS^-$ in solution, then $HS^-$ reacts with $H^+$ and produce $H_2S$ (Hosoki et al., 1997). It has been also reported that $H_2S$ is in equilibrium with $HS^-$ in the cell (Rausch and Wachter, 2005). In the present study, $Na_2S$, $Na_2SO_4$ and $Na_2SO_3$ were used in the same experimental design to verify the role of $H_2S$ in decreasing NaHS induced lipid peroxidation during cold stress. The results indicated that $Na_2S$, $Na_2SO_4$ and $Na_2SO_3$ pretreatment are not able to decrease the MDA and other aldehydes contents (Table 1). Therefore, we concluded that $H_2S$ or $HS^-$, rather than other sulfur-containing compounds, are responsible for protective roles of NaHS during cold stress. There has been no previous report on the direct role of $H_2S$ in the reduction of lipid peroxidation and electrolyte leakage. However, Shi et al. (2014) has shown that $H_2S$ decreases the lipid peroxidation through other signaling pathways such as nitric oxide (Shi et al., 2014).

### *$H_2O_2$ content and antioxidant enzyme activity*

$H_2O_2$ is one of the main forms of ROS in biological systems that accumulates under chilling stress and can be used as an index for oxidative damage (Liu et al. 2010). In the present study, $H_2S$ pretreatment resulted in a significant reduction in the $H_2O_2$ content in plants under stress condition.

Hancock and Whiteman (2014) reported that $H_2S$ has some roles to regulate the levels of other reactive species such as ROS and nitric oxide as well as to ensure that the over-accumulation of such compounds, or their effects, is not causing damage to cells and tissues (Hancock and Whiteman, 2014). Consistent with our results, it has also been reported that $H_2S$ donor increases the activity of ROS scavenging enzymes that reduces $H_2O_2$ and leads to a reduction in lipid peroxidation and membrane damage (Zhang et al., 2010a).

Results of the present study showed that $Na^+$ or sulfur-containing compounds except NaHS are not able to decrease $H_2O_2$ content in cucumber seedlings exposed to cold stress (Table 1). Therefore, we conclude that $H_2S$ can direct or indirectly decrease $H_2O_2$ content in plants which were exposed to cold stress. Conversion of $O^{\bullet 2}$ to $H_2O_2$ and $O_2$, through SOD activity is an important step in protecting the cells from oxidative damages. Efficient scavenging of $H_2O_2$ is an important step for normal plant metabolism. CAT, GPX and APX enzymes are important $H_2O_2$ scavenger enzymes. In the present study, $H_2S$ pretreatment increased the activity of these antioxidant enzymes in cold stressed plants when compared with non-pretreated plants (Fig. 3 and 7).

Similarly, elevated activity of SOD, POD, CAT, and APX due to $H_2S$ pre-treatment have also been reported in wheat exposed to copper toxicity (Zhang et al., 2010a). It has been reported that treatment of Bermuda grass with SNP and NaHS, under cadmium stress, decreases $H_2O_2$ and $O_2^-$ contents and elevates antioxidant enzymes (e.g. SOD, CAT, POD and GR) activity (Shi et al., 2014).

***Suger, prolin and GSH content***

In this study foliar application of exogenous $H_2S$ on cucumber plants significantly increased the sugar content in the cucumber leaves (Fig. 4). It has been reported that sugars might protect plant cell membranes during cold-induced dehydration, replacing watermolecules in establishing hydrogen bonds with lipid molecules (Uemura et al., 2003; Ruelland et al., 2009). Moreover, carbohydrates may also act as scavengers of ROS and contribute to improved membrane stabilization (Bohnert and Sheveleva 1998). It has been shown that sugar signaling is also closely associated with hormone signaling and could regulate the growth, development and stress responses in plants (Zeng et al., 2011). Moreover, accumulation of osmolytes (proline, sucrose and soluble total sugars) under stress conditions is essential to protect plants through balancing osmotic pressure and modulating cell membrane stability which is very important for cold stress resistance. Exogenous NaHS application in Bermuda grass has also lead to significant accumulation of osmolytes under NaCl, drought and cold stress conditions (Shi et al., 2013), which was similar to the finding of present study. The positive modulation of exogenous $H_2S$ donor (NaHS) in osmolyte accumulation might also contribute to enhanced stress tolerance.

It has been reported that proline accumulation leads to improvement of tolerance to cold stress. Proline plays multiple roles in plant stress tolerance. For instance, it acts as a mediator of osmotic adjustment, a stabilizer of proteins and membranes, inducer of osmotic stress-related genes, and scavenger of ROSs (Maller et al., 2002). Although there are no previous reports on modulation of proline metabolism by $H_2S$, many studies have indicated that other gas signal molecules such as nitric oxide are involved in the metabolism of proline under stress conditions (Lei and Li, 2007; Lopez-carrion et al., 2008., Farooq et al., 2009). Counteraction between $H_2S$ and NO were also reported by some studies before (Lisjak et al., 2010; Wang et al., 2012). Therefore, the modulation of proline by $H_2S$ might be involved in nitric oxide pathway, a mechanism that needs further investigation.

GSH is another important compound of plant antioxidant system. The cellular content of GSH can be determined by γ-ECS and GR, which are the enzymes for glutathione biosynthetic and recycling pathways (Shan et al., 2011). In previous studies, it has been reported that signal molecules, such as $Ca^{2+}$, ABA, jasmonic acid, nitric oxide and $H_2O_2$ can regulate glutathione metabolism and have important roles in stress responses (Ai et al., 2008,

Hu et al., 2008). In the present study, data showed that $H_2S$ increased glutathione content under stress condition (Fig. 6), this function of $H_2S$ may be performed through the regulation of GSH metabolism. This increase has an important role in defense against oxidative stress in cucumber, although the signal transduction of $H_2S$ in regulating glutathione metabolism remains unclear.

Although the mechanism of exogenous $H_2S$ on alleviating abiotic stress is still not completely understood, interaction between nitric oxide and $H_2S$ has been reported in plants exposed to several stress conditions such as heat (Li et al., 2013b), cadmium (Li et al., 2012a) and salt (Wang et al., 2012) stress. In addition, it has been shown that nitric oxide signal could be blocked by both nitric oxide and $H_2S$ scavengers and inhibitors, while $H_2S$ signal was only specifically blocked by $H_2S$ scavenger and inhibitors, indicating that nitric oxide can act as the up-stream signal of $H_2S$ (Shi et al., 2014). However, application of nitric oxide scavenger decreases the protective effects of $H_2S$; therefore some effects of $H_2S$ are most likely trough nitric oxide signaling pathway. In the present study some physiological and biochemical effects of $H_2S$ in alleviating of chilling stress may be related to nitric oxide signaling pathway. However, mechanism of nitric oxide involvement in cold stress tolerance needs to be future investigated.

## Acknowledgment

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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