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Hydrogen sulfide protects coriander seedlings against copper stress by regulating the ascorbate-glutathione cycle in leaves

Elham Asadi Karam and Batool Keramat*
Biology Department, Shahid Bahonar University of Kerman, Kerman, Iran
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Abstract:

Heavy metals are the cause of major abiotic stresses in plants and a principal contributor to environmental pollution in recent decades. This study investigated the effects of exogenous hydrogen sulfide on the ascorbate-glutathione cycle in the leaves of coriander seedlings under copper stress. Results showed that copper stress not only reduced APX and GR activities but also decreased leaf AsA, DHA, and GSH contents. Pretreatment with sodium hydrosulfide (NaHS), a hydrogen sulfide (H_2S) donor, was observed to enhance both GR activity and AsA, GSH, and DHA contents under copper stress. Moreover, the pretreatment decreased the malondialdehyde content and electrolyte leakage induced by copper stress in plants. Based on the results obtained, it was hypothesized that exogenous hydrogen sulfide alleviates oxidative damage under copper stress by regulating the ascorbate-glutathione cycle and, further, that H_2S plays an important role in the acquisition of copper stress tolerance in coriander seedlings. Exogenous hydrogen sulfide is, therefore, identified as an agent with the potential to be used as a regulator to improve crop tolerance under copper stress.

Keywords: Copper stress, Coriander, Hydrogen sulfide, Ascorbate, Glutathione.

Abbreviations: APX: Ascorbate Peroxidase; ASA: Ascorbate; DHA: Dehydroascorbate; GR: Glutathione Reductase; GSH: Glutathione; H_2S: Hydrogen Sulfide; MDA: malondialdehyde; NaHS: Sodium Hydrosulfide.

Introduction:

Plants depend on adequate amounts of copper for their normal growth. The metal is an essential redox component participating in a wide variety of processes, including the electron transfer reactions of respiration and photosynthesis or the detoxification of superoxide radicals (Fox and Guerinot, 1998). However, excess copper can induce changes in the photosynthetic and respiratory processes, enzyme activities, as well as DNA and membrane integrity (Hazen *et al*., 1988; Vinit-Dunand *et al*., 2002; Alaoui-Sossé *et al*., 2004; Lombardi and Sebastiani, 2005). Another important feature of copper stress is the induction of oxidative damage to plants by inducing reactive oxygen species (ROS) accumulation (Demirevska-Kepova *et al*., 2004). If not effectively and rapidly removed from plants, ROS can damage a wide range of cellular macromolecules such as lipids, enzymes, and DNA (Contreras *et al*., 2009). Under non-stress conditions, ROS are removed by non-enzymatic and enzymatic antioxidants, whereas during a stress, the production of ROS exceeds the capacity of the antioxidative systems (Noctor and Foyer, 1998). The non-enzymatic antioxidants include ascorbate (ASC) and GSH, the two main constituents of the ASC-GSH cycle which detoxify H_2O_2 in chloroplasts and cytosol (Gill and Tuteja, 2010; Potters *et al*., 2010). Scavenging H_2O_2 by ascorbate peroxidase (APX) is the first step in the ASC-GSH cycle, which maintains the ASC pool in its reduced form (Potters *et al*., 2010). Glutathione reductase (GR) is the key enzyme for maintaining the GSH pool (Rennenberg, 1982).

It has been shown that hydrogen sulfide (H_2S) can act as the third gaseous signaling molecule in animals after nitric oxide (NO) and carbon monoxide (CO) (Hosoki *et al*., 1997). In plants, NO and CO have already been identified as signaling molecules involving an antioxidative defense effect (Delledonne, 2005; Huang *et al*., 2006). Recently, it has also been documented that H_2S can promote root organogenesis (Zhang *et al*., 2009a) and seed germination (Zhang *et al*., 2010a). Moreover, there is increasing evidence showing that H_2S is involved in plant antioxidative response to stress conditions (Zhang *et al*., 2010b). However, whether H_2S regulates the ascorbate-glutathione cycle in coriander plants under copper stress remains to be known. In this study, we investigated the malondialdehyde content, electrolyte leakage, the enzyme activities involved in the ascorbate-glutathione cycle, and the AsA, GSH, total ascorbate, and total glutathione contents in the leaves of coriander seedlings exposed to copper stress induced by 100 µM of $CuSO_4$ solution. The objective of the study was two-fold: to elucidate whether H_2S regulates the ascorbate-

***Corresponding Author, Email: Bkeramat@uk.ac.ir**

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glutathione cycle at both molecular and physiological levels under copper stress, and to provide new knowledge on the antioxidant metabolism in plants under copper stress.

Materials and methods:

Plant material, growth conditions, and treatment procedures: The seeds of *Coriandrum sativum* were washed with sterile distilled water and transferred to plastic pots containing perlite. A fresh nutrient solution (pH 5.7 ± 0.1) was prepared for irrigation every time from the stock solutions (Hoagland and Arnon, 1950). The plants thus grown were kept in a greenhouse with a photoperiod of 16/8 h light/dark and 14 K lux light intensity at 28/18 ºC day/night and a relative humidity of 50–60%.At the three-leaf stage, the seedlings were exposed to three levels (0, 100, and 200 µM) of sodium hydrosulfide (Sigma) used as a foliar spray for 72 hours (once a day). After 72 h of initial pretreatment, the plants were irrigated with the half strength Hoagland's solution containing copper sulfate salt ($CuSO_4$) (100 µM) for 6 days (Asadi karam *et al*., 2015). At the end of the experiment, the leaves of the plants were harvested, immediately frozen in liquid nitrogen, and stored at –80 °C until analysis.

Membrane damage determination: The level of lipid peroxidation in plant tissues was measured by determining the malondialdehyde (MDA) content using thiobarbituric acid (Heath and Packer, 1968). The procedure described in Ben Hamed *et al*. (2007) was used to determine leaf electrolyte leakage which was then used to calculate membrane stability.

Measurement of ascorbate (ASA), dehydroascorbate (DHA), and GSH contents: The plants were homogenized with 5% metaphosphoric acid at 4 °C. The homogenate was then centrifuged at 20,000 g for 15 min at 4 °C and the supernatant was collected for the analysis of ascorbate and glutathione. ASA and DHA were determined according to the method of Kampfenkel *et al*. (1995). Briefly, total ascorbate was determined after reduction of DHA to ASC with dithiothreitol and DHA concentration was estimated from the difference between the total ascorbate pool (ASC plus DHA) and the quantity of ASC produced.

GSH content was determined using the spectrophotometric method of Ellman (1959). For this purpose, GSH was oxidized in 2.6 ml of a sodium phosphate buffer (pH 7.0) containing 0.2 ml of a sample extract and 0.2 ml of 6 mM 5, 5'-dithiobis-(2-nitrobenzoic) acid (DTNB). The absorbance was monitored at 412 nm. GSH content was calculated from a standard curve constructed using GSH over the range 0–100 µM.

Antioxidant enzyme activity: For protein extraction and analysis, the extracts from the frozen samples prepared in a 50 mM potassium phosphate buffer (pH 7) containing 1mM phenylmethane sulfonyl fluoride (PMSF), 1 mM sodium ethylene diaminetetraacetic acid (Na_2EDTA), and 1% (m/v) polyvinylpyrrolidone (PVP) were centrifuged at 15000 *g* at 4 °C for 15 min. The supernatants collected were used for the estimation of protein content and enzyme activities. The total protein content was measured according to the method of Bradford (1976) using the bovine serum albumin as the standard.

The ascorbate peroxidase (APX; EC 1.11.1.11) was assayed by monitoring the decrease in absorbance at 290 nm due to ASC oxidation (Nakano and Asada, 1987). The reaction mixture contained 50 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 0.15 mM H_2O_2, 0.5 mM ASC, and 0.15 cm^3 of the enzyme extract. The activity of APX was calculated using ε = 2.8 mM^{-1} cm^{-1}. One unit of APX activity was defined as the amount of enzyme that decomposed 1 mmol of ascorbate per minute.

The activity of glutathione reductase (GR; EC1.6.4.2) was determined following the decrease in absorbance at 340 nm associated with the oxidation of NADPH (Foyer and Halliwell, 1976). The assay contained 50 mM Tris- HCl (pH 7.8), 150 µM NADPH, 500 µM oxidized glutathione (GSSG), and 0.05 ml of the enzyme extract. One unit of GR was defined as the amount of enzyme that oxidized 1 µmol of NADPH per minute.

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was determined based on the inhibition of nitroblue tetrazolium (NBT) reduction to furmazone at pH 7.0 (Giannopolitis and Ries, 1977). The reaction mixture contained 50 mM of the potassium phosphate buffer (pH 7.0), 0.1 mM Na_2EDTA, 75 µM riboflavin, 13 mM methionine, and 0.05 ml of the enzyme extract. One unit of SOD activity was defined as the amount of the enzyme that inhibited 50% NBT photoreduction.

Statistical analysis: Data analysis was accomplished by the one-way ANOVA using SPSS software, Version 18 for Windows. The Duncan's multiple range test (DMRT) was used to separate the means for significant treatment ($p \geq 0.05$). Values were reported as means of three replicates ± SE.

Results and Discussion:

To investigate the likely effects of H_2S on copper tolerance in coriander seedlings, the effects of pretreatment with NaHS on the malondialdehyde content and electrolyte leakage were studied in leaves under copper stress. The results showed that copper stress significantly increased the malondialdehyde content and electrolyte leakage in coriander leaves, confirming similar results reported elsewhere (Saha *et al*., 2011; Mohanpuria *et al*., 2007). Pretreatment with NaHS led to significant decreases in the two parameters induced by copper stress (Figs. 1A, 1B). No differences were, however, observed between the control samples and those pretreated with exogenous H_2S alone with respect to their malondialdehyde content or electrolyte leakage. These results suggest that H_2S plays an important role in the acquisition of copper stress tolerance in coriander seedlings.

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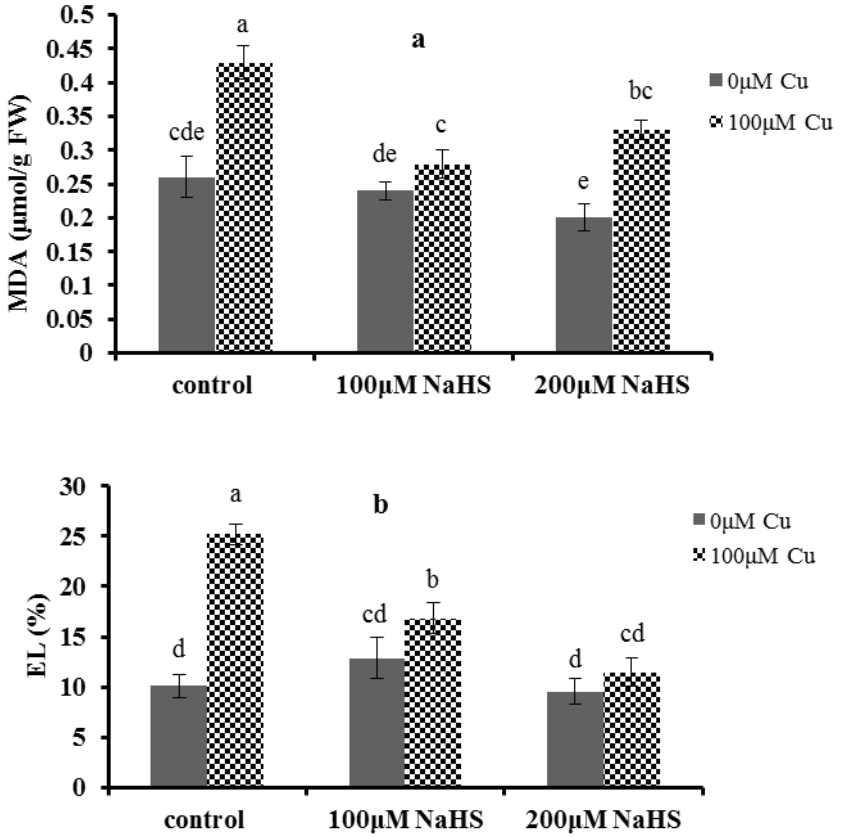

Figure 1- Effects of copper (Cu) and sodium hydrosulfide (NaHS) application on malondialdehyde content (A) and electrolyte leakage (B) in *Coriandrum sativum*. Values are means ± SE (*n* = 3). In the individual column, bars with different letters are statistically different (*P* < 0.05) according to the Duncan's multiple range tests.

The plants pre-treated with NaHS in the present study exhibited a higher tolerance to Cu toxicity. It has been documented that AsA has an important role to play in counteracting the effects of stress conditions. While tolerance to certain heavy metals in some plants is associated with increases in both APX and GR activities (Madhava Rao and Sresty, 2000), we observed a decrease in GR activity under Cu stress (Table1) as a result of NaHS application. Compared to the control, pretreatment with NaHS alone led to a significant increase in GR activity but had no effect on APX activity in leaves. These results suggest that application of exogenous H_2S is capable of increasing GR activity under copper stress. Previous study has shown H_2S to increase APX activity in the root tip of *Pisum sativum* (Li *et al*., 2010). Zhang *et al.* (2010a, b) also reported that H_2S enhanced APX activity in wheat under chromium, aluminum, and osmotic stresses. However, Zhang *et al*. (2008) reported that H_2S did not affect the APX activity in wheat under copper stress. This finding is in agreement with ours in the present study that H_2S has no effect on APX activity in coriander under copper stress. Reduced SOD activity under Cu stress and the significantly increased SOD activity due to NaHS application, compared to the situation with Cu stress alone (Table1), greatly contribute to the scavenging of the superoxide radical to H_2O_2 (Alscher *et al*., 2002). Recent studies have demonstrated that the overexpression of mitochondrial Mn-SOD and chloroplastic Cu/Zn-SOD enhance tolerance to stress in transgenic *Arabidopsis thaliana* (Wang *et al.*, 2004) and in transgenic *Nicotiana tabacum* (Badawi *et al.*, 2004), respectively. Similar results have been reported in pigeon pea seedlings (Madhava Rao and Sresty, 2000).

Means followed by similar letters in each column are not significantly different at p=5% based on Duncan's multiple range tests Ascorbate and glutathione are the two major non-enzymatic antioxidant molecules in plants with significant contributions to plant antioxidant machinery and tolerance to abiotic stresses (Gill and Tuteja, 2010; Potters *et al*., 2010). High ascorbate and glutathione redox ratios are necessary to achieve optimal metabolism and to promote tolerance to abiotic stress (Foyer and Noctor, 2005; Fotopoulos *et al*., 2010) while low ascorbate redox ratios result in increased sensitivity to oxidizing agents (Fotopoulos *et al*., 2006). Compared to the control, GSH and ASC accumulations decreased under the treatment with 100 µM Cu used in the present study. Addition of NaHS was found to have different influences on leaf ASC and GSH contents while pretreatment with NaHS significantly increased AsA, GSH, and DHA contents under copper stress (Fig. 2). This might have been due to the greater reductions in ascorbate and glutathione contents as also confirmed by Shan *et al*. (2011, 2012) who observed similar trends in the ASC and GSH

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Table 1- Effects of NaHS application on APX, SOD, and GR activities in coriander leaves under copper stress

Treatment	GR activity (U/mg protein)	APX activity(Unit/mg protein)	SOD activity (U/mg protein)
Control	1.61^{c}	58.36^{cd}	20.21^{d}
Cu 100 µM	1.12^{d}	45.66^{e}	17.13^{e}
NaHS 100 µM	1.45^{bc}	51.71^{d}	22.45^{cd}
NaHS 200 µM	1.55^{bc}	63.53^{ab}	28.64^{c}
Cu100 µM +NaHS 100 µM	2.23^{ab}	59.68^{cd}	32.87^{b}
Cu100 µM +NaHS 200 µM	2.64^{a}	64.97^{abc}	35.73^{a}

In the individual column, bars with different letters are statistically different ($P < 0.05$) according to the Duncan's multiple range tests.

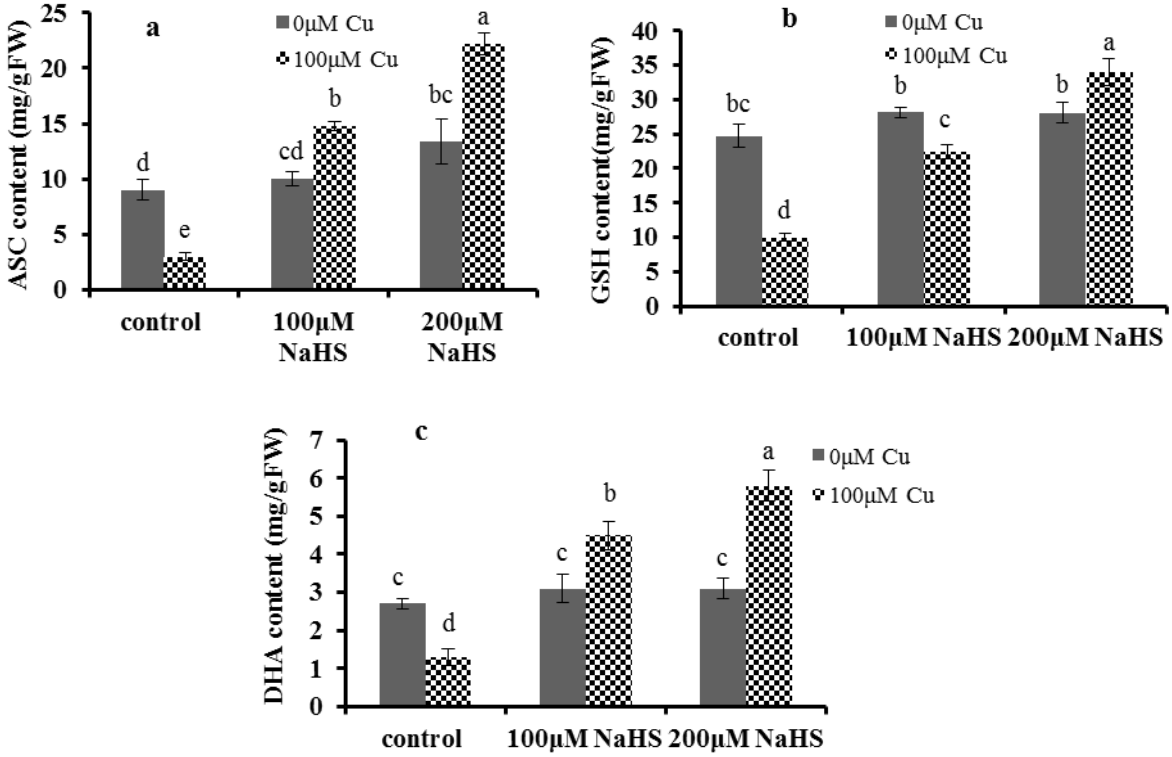

Figure 2-Effects of copper (Cu) and sodium hydrosulfide (NaHS) on ASC (A), DHA (B), and GSH (C) contents in *Coriandrum sativum*. Values are means ± SE ($n = 3$). In the individual column, bars with different letters are statistically different ($P < 0.05$) according to Duncan's multiple range tests.

contents in wheat pretreated with NaHS and subsequently subjected to water and copper stresses. Meanwhile, pretreatment with NaHS alone led to significant increases in leaf AsA, GSH, and DHA contents, as compared to the control.

Another compound of great significance to the plant antioxidant system is GSH. The cellular GSH content may be determined by GR which are the enzymes of the recycling pathway. The results of our study showed that H_2S may regulate the glutathione cycle by enhanced GR activities and GSH content under copper stress. In agreement with our experimental results, H_2S has been reported to induce GSH accumulation in rice suspension cell (Ma, 2007). Moreover, our findings indicate that H_2S increases GR activity under copper stress.

Conclusion:

The results of this study show that the application of copper causes toxicity in such plants as coriander as evidenced by their increased lipid peroxidation and electrolyte leakage. Increasing endogenous treatment of H_2S was, however, found to reduce plant MDA content due to the enhanced GSH levels observed. This suggests that endogenous H_2S prevents the damaging effects of copper stress as a result of increasing GSH and decreasing MDA contents. Our results also imply that exogenous hydrogen sulfide alleviates oxidative damages by regulating the ascorbate-glutathione cycle under copper stress so that H_2S might be claimed to play an important role in the acquisition of copper stress tolerance in coriander seedlings.

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