Introduction

For a long time, hydrogen sulfide (H2S) was considered a toxic compound, but recently it has been found H2S (at low concentrations) play an essential function in physiological processes (Olas 2014). H2S controls seed germination, root development, stomatal aperture, flower senescence, and pathogen challenge (Hancock and Whiteman 2014). Many plant species can generate H2S, suggesting that it may be an endogenous chemical, suitable to act as a signaling molecule (Li 2013). H2S can be considered as a nutrient; thus exposure to H2S increases cysteine for synthesis of proteins and all other cellular components necessary for growth. Treatment with H2S increases the availability of reduced sulfur for synthesis of glutathione (GSH) and the dominant player in defense against a wide range of stresses that increase the cellular capacity to remove ROS (Caldewood and Kopriva 2014), therefore it can alleviate oxidative stress in plants. For example, It has been shown that H2S plays a role in plant adaptive responses to salinity, drought, high temperature and heavy metal exposure (Li 2013).

Ozone depletion leads to UV radiation on the earth surface and has deleterious effects on living organisms, particularly plants (Ulm and Nagy 2005). Numerous studies have shown that enhanced UV-B radiation significantly affects morphological, physiological, and biochemical processes of many plant species (Kataria et al. 2014). Exposure of plants to UV-B radiation, causes ROS production, which can impair cellular processes and cause their dysfunction and oxidative stress (Nawkar et al. 2013). Oxidative stress causes reversible or irreversible modifications of bio-molecules such as proteins, polynucleic acids, carbohydrates, and lipids (Gill and Tuteja 2010). Further, plants develop strategies to alleviate the UV-induced damage. Plants procedures include the shielding of the organelle by inducing the accumulation of UV-absorbing phenolic compound and repair of DNA damage via DNA photolyase (Jenkins 2009). Plants have also developed complex antioxidant defense systems involving several enzymes and metabolites, to scavenge excess ROS produced under UV-B stress (Hasanuzzaman et al. 2012).

Borage (Borago officinalis L.), which was chosen in our research, is an important medicinal plant of the Mediterranean region. Borage is an annual plant that grows wild in the Mediterranean countries and has been cultivated elsewhere. This plant, recently, has been the subject of increasing agricultural interest because of the potential market for γ-linolenic acid (Gupta and Singh 2010).

In this study, we used NaHS, a donor of H2S, on borage seedlings, to investigate the role of H2S in the alleviation of UV-B radiation damages focusing on oxidative parameters.

Materials and methods

Plant material, culture conditions, and stress treatments

Borago officinalis L. seed was supplied by Pakan Bazar Company, Isfahan, Iran. NaHS was purchased from Merck Company and used as H2S donor. UV-B radiation was provided by a UV-B lamp LF-215 model, made in uvitec (Cambridge) company with 312 nm wavelengths. The seed of borage grew in the greenhouse with 16/8 h (light/dark), at 23°C and with 50% relative humidity. The seedlings were irrigated with water and half-strength Hoagland solution tertian. After 20 days, plants were divided in to 3 groups with 4 replicates.
and NaHS (acting as an exogenous H₂S donor) with different concentration solutions and same volume (40 and 120 μM, 0.1% Tween-20 was used as a surfactant, pH = 6–6.5) was sprayed on leaves of borage plants for 5 days (one group sprayed with distilled water as control). After this period, the pre-treated plant was exposed to UV-B radiation for 7 days (30 min daily). Then leaves of all groups were harvested and immediately frozen in liquid nitrogen and stored at −80°C for Biochemical analysis.

Lipid peroxidation, protein oxidation, and hydrogen peroxide (H₂O₂) assay

The level of lipid peroxidation, as an indicator of membrane damage, was measured in terms of malondialdehyde (MDA) content, according to Heath and Packer (1968).

The amount of protein oxidation was estimated by the reaction of carbonyl groups with 2,4-dinitrophenylhydrazine (2,4-DNPH), as described by Levine et al. (1994). For this purpose, after reaction of carbonyl groups with 2,4-DNPH, the carbonyl content, according to Heath and Packer (1968), was calculated by absorbance at 370 nm, using an extinction coefficient of 22 mM⁻¹ cm⁻¹ for aliphatic hydrazones and expressed as nanomole of carbonyl per milligram of protein.

H₂O₂ content was measured calorimetrically after reaction with potassium iodide (KI) according to the method of Alexieva et al. (2001).

Enzymes extraction and assays

For antioxidant enzymes activities assay, leaves (500 mg) were homogenized in 50 mM potassium phosphate buffer (pH 7.0) containing 1% soluble PVP, 1 mM EDTA and 1 mM PMSF, with the addition of 1 mM ascorbate in the case of APX assay. The homogenate was centrifuged at 20,000 × g for 20 min and the supernatant used for test of protein content (Bradford 1976) and the activity of enzymes.

Catalase (CAT) activity was determined spectrophotometrically by following the decrease of absorbance of H₂O₂ within 30s at 240 nm using the extinction coefficient of 40 mM⁻¹ cm⁻¹ for H₂O₂ (Dhindsa et al. 1981). CAT activity was expressed as unit per mg of protein.

Ascorbate peroxidase (APX) activity was estimated according to the method of Nakano and Asada (1981) following a decrease in absorbance at 290 nm in a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.0), 0.1 mM Na₂EDTA, 5 mM of ascorbate, 0.5 mM of H₂O₂ and enzyme extract. Enzyme activity was determined using the extinction coefficient of 2.8 mM⁻¹ cm⁻¹ and was calculated as the amount of enzyme required to oxidize 1 μM of ascorbate per min per mg protein.

The GPX activity was determined using the method of Zhang et al. (2005) following the formation of tetraguaiacol by monitoring the increase in absorbance at 470 nm for 3 min (extinction coefficient of 25.5 mM⁻¹ cm⁻¹). Activity was expressed as units (μM of oxidized guaiacol per min) per mg of protein.

GSH assays

GSH content was estimated following a modified method, according to Ellman (1959). 200 mg of the leaves were homogenized in 4 ml of 15% meta-phosphoric acid and centrifuged at 10000 × g for 30 min at 4°C. Aliquot of 0.2 ml of the supernatant was mixed with 2.6 ml 150 mM sodium 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. GSH content was calculated using the standard curve of GSH.

Flavonoids analysis

For total flavonoids analysis, 500 milligrams of the leaves was extracted with absolute ethanol and water (1:1, v/v) in 10% ratio (0.5 g leaves was extracted with 5 ml EtOH, H₂O) for 4 h in room temperature. Extraction was carried out by use of vortexing for 30 min intervals. The mixture was centrifuged at 5000 × g for 20 min at room temperature, and the supernatant was used for high pressure liquid chromatographic (HPLC) analysis. Injection volume was 20 μl and elution performed with a flow rate of 0.8 ml min⁻¹. The C18 column was used (Zorbax 300SB). The solvents comprised water adjusted to pH = 2.5 with orthophosphoric acid (A) and acetonitrile (B) which were mixed with a linear gradient starting with 100% A, decreasing to 91% over the next 12 min to 87% over the next 8 min and to 67% over the next 10 min. After holding the solvent at this composition for 2 min, A was decreased to 57% over the next 10 min, and then held at this level until the end of the 60 min analysis. Peaks were detected at 340 nm (Campos et al. 1997).

Statistical analysis

All determinations were carried out in three triplicates, and data were subjected to analysis of variance. Analysis of variance was performed using the ANOVA procedure. Statistical analyses were performed according to the SPSS software. Significant differences between means were determined by Duncan’s multiple range tests. P values less than 0.05 were considered statistically significant.

Results

Effects of exogenous H₂S and UV-B radiation on MDA content, carbonyl groups, and Hydrogen peroxide

The results showed that when the Borage plant was exposed to UV-B radiation, the content of MDA, protein oxidation, and H₂O₂ production, significantly increased. However, NaHS pre-treatment significantly reduced the MDA content, carbonyl groups, and H₂O₂ in those plants which were under UV-B stress. The higher NaHS concentration (120 μM) was more effective than lower concentration to reduce the level of oxidative parameters (Figure 2). Application of NaHS in control plants also decreases the H₂O₂ content.

Effects of exogenous H₂S and UV-B radiation on the activities of CAT, GPX, APX, and GSH content

To investigate the protective mechanism of H₂S the activities of several antioxidant enzymes were measured. Under UV-B stress, activities of CAT, GPX, and APX did not significantly change but when NaHS was sprayed CAT activity in the lower level of H₂S (40 μM NaHS), and GPX and APX in a higher level of H₂S (120 μM NaHS) significantly increased (Figure 2). When plants were treated with different
concentration of exogenous H$_2$S and exposed to UV-B stress, the content of GSH increased in the leaves of B. officinalis plants (Figure 3). Pre-treatment with NaHS also increased APX activity and GSH content in control plants as well.

**Effects of exogenous H$_2$S and UV-B radiation on flavonoid concentration**

In those plants which were exposed to UV-B radiation, enhancement of Flavonoid concentration was observed. In these plants, which were pre-treated with a higher level of H$_2$S and exposed to UV-B radiation, Flavonoids significantly decreased (Figure 1).

**Discussion**

UV-B radiation has the highest energy of any part of the daylight spectrum and has the potential to damage the plants by producing reactive oxygen species (Jenkins 2009). Recently, many studies confirmed that H$_2$S plays an important role in responses against abiotic stresses (Gua et al. 2016). For example, H$_2$S was involved in responses to salinity, drought, and Cd exposure (Jin et al. 2011; Lai et al. 2014).

In this research, exposure to UV-B stress caused considerable Flavonoids concentration enhancement, but pre-treatment with H$_2$S significantly decreased them (Figure 1). It has been reported that the most consistent response to UV-B exposure is an increase in foliar concentrations of UV-B absorbing compounds (Searles et al. 2001). These compounds absorb effectively in the UV-B region while having little absorption in the visible region, and are located predominantly in the vacuoles of epidermal cells. In higher plants, UV-B absorbing compounds include a large number of phenylpropanoids, particularly flavonoids (Robberecht and Caldwell 1983). Increases in UV-B absorbing compounds appeared to protect DNA and PSII from UV-B. UV-B-responsive flavonoids, in general have the potential to reduce the oxidative damage caused by short solar wavelengths, in addition to reducing the risk of ROS generation by attenuating the penetration of UV-B radiation to sensitive leaf targets (Agati et al. 2009). Mutants with reduced phenolic synthesis have been shown to exhibit increased sensitivity to UV-B compared with the wild type (Xu et al. 2008). While in this study, the overproduction of H$_2$O$_2$, MDA, and carbonyl groups, a product of protein oxidation, were observed in the stressed plant (Figure 2) which may suggest that flavonoid contents were not sufficient for filtering UV-B, and overproduction of ROS was attributable to oxidative damage. In our experiment, flavonoids content reduced with a higher concentration of NaHS that seems to be the reason for reinforcement of the first antioxidant system in the present of H$_2$S. It was reported that the biosynthesis of flavonoids increase more in stress-sensitive species than stress-tolerant species which suggests stress-sensitive species are less effective in the first line of defense against ROS under stressful conditions and are subsequently exposed to a more severe oxidative stress. Thus, flavonoids may constitute a ‘secondary’ antioxidant system that is activated as a consequence of the depletion of antioxidant enzyme activities (Agati et al. 2012).

Overproduction of MDA and carbonyl groups have been reported in abiotic stress, for example, drought stress in

![Figure 1](image-url)
tomato (Nasibi and Kalantari 2009) and cold stress in Pistachio (Nasibi et al. 2013). ROS generated by stress can be removed by ROS-scavenging enzymes systems, thereby maintaining a balanced redox state (Mittler 2006). We found that pre-treatment of Borage plants with H2S increased the activity of antioxidant enzymes especially APX and GPX in plant against UV-B stress (Figure 3) while MDA content, carbonyl groups, and H2O2 was decreased in these Pre-treated groups (Figure 2). It has been reported that, the role of H2S was characterized by increased antioxidant enzymes activities, which is beneficial for plant performance under stresses (Li et al. 2014; Ahmad et al. 2019). We found that pre-treatment of Borage plants with H2S increased the activity of antioxidant enzyme especially APX and GPX in plant against UV-B stress (Figure 3) while MDA content, carbonyl groups, and H2O2 was decreased in these Pre-treated groups (Figure 2). It has been reported that, the role of H2S was characterized by increased antioxidant enzymes activities, which is beneficial for plant performance under stresses (Li et al. 2014; Ahmad et al. 2019). It was shown that, H2S increased the activity of antioxidative enzymes by their up-regulating in stress condition (Zhang et al. 2009; Wang et al. 2012; Gua et al. 2018; Jiang et al. 2019) therefore ROS production is limited, hence alleviation of damage to cellular components is possible. Fang et al. (2014) reported that use of exogenous H2S decrease ROS production by inhibiting NADPH oxidase in Cr stress. Aghdam et al. (2018) also reported that the addition of exogenous H2S by increasing the activity of antioxidant enzymes reduced the accumulation of H2O2 during cold storage of hawthorn fruits.

In this study, pre-treatment of plants with NaHS also significantly enhanced the GSH content of the stressed plant. Glutathione is a major non-enzymatic antioxidant molecule in plants, with significant contribution to the plant antioxidant machinery and tolerance to abiotic stresses (Srivalsi and Khanna-Chopra 2008). Multiple actions of glutathione in plant metabolism concern signaling of sulfur status, resistance to xenobiotics, heavy metal tolerance, and pathogen response. It also maintains redox homeostasis in cells (Zagorchev et al. 2013). The change in the ratio of its reduced (GSH) to oxidized (GSSG) form during the degradation of H2O2 is important in specific redox signaling pathways (Hameed et al. 2014). There is a connection between H2O2 concentration and GSH/GSSG ratio, thus increase of H2O2 content leads to a decrease of GSH pool in the cell (Rouhier et al. 2008). The ability of plants to increase non-protein thiols, including GSH, maybe the reflection of tolerance to different stresses (Zagorchev et al. 2013). In this study, the increase of GSH content concomitant with APX activity showed the role of ascorbate–glutathione cycle, which is likely induced by H2S. Decrease of H2O2 content in pre-treated plants probably proves this hypothesis. It has been reported that, H2S can promote expression of essential GSH biosynthetic genes (Christou et al. 2013) and up-regulation of the ascorbate–glutathione cycle (Singh et al. 2015).

**Conclusion**

In summary, our results demonstrated that UV-B stress caused the oxidative damage in borage leaves through the excessive generation of ROS, but the application of exogenous H2S alleviated the UV-B damages by increasing antioxidant enzyme activities and GSH content. Decrease of flavonoid content in leaves of stressed plants by H2S may confirm this idea.
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Disclosure statement
No potential conflict of interest was reported by the authors.

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