Hydrogen Sulfide Modulates Salinity Stress in Common Bean Plants by Maintaining Osmolytes and Regulating Nitric Oxide Levels and Antioxidant Enzyme Expression

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Abstract
The purpose of the present study is to investigate the role of hydrogen sulfide (H2S), in improving resistance to common bean salt stress. Method shows that common bean seeds were soaked in water and in two concentrations of sodium hydrosulfide (50 and 100 µM) for 8 h. After 25 days from sowing, the pots were irrigated with water and with two concentrations of NaCl (75 and 150 mM) until the end of the experiment. Results revealed that H2S relieved salt stress by decreasing growth inhibition and photosynthetic characteristics, and increasing osmolyte contents (proline and glycine betaine). Furthermore, H2S reduced oxidative damage by lowering lipid peroxidation, electrolyte leakage, and reactive oxygen species production such as hydrogen peroxide, hydroxyl radicals, and superoxide anion by increasing non-enzymatic antioxidants such as ascorbic acid and glutathione, as well as enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POX), ascorbate peroxidase (APX), glutathione reductase (GR), and nitrate reductase (NR). Meanwhile, salt stress and H2S application increased the endogenous level of H2S, which was accompanied by an increase in nitric oxide concentration. H2S, in particular, maintained sodium (Na+) and potassium (K+) homeostasis in the presence of excess NaCl. In general, H2S effectively reduced oxidative stress in common bean plants by increasing relative expression levels of copper-zinc superoxide dismutase (Cu-ZnSOD), CAT, and glutathione S-transferase (GST). Applying H2S to common bean plants could protect them from salinity stress by maintaining the Na+/K+ balance, boosting endogenous H2S and nitric oxide levels, and preventing oxidative damage by increasing antioxidant activity.

Keywords Antioxidant enzymes · Ascorbic acid · Glutathione · Gene expression · Hydrogen sulfide · Osmolytes · Nitric oxide

1 Introduction
Soil salinization has become one environmental and socio-economic issue globally, which is expected to be exacerbated by projected climatic change. Due to highly complex processes influencing salinization, determining how climate change influences naturally occurring soil salinization dynamics has scarcely been addressed (Hassani et al. 2021). More than 20% of the world’s soils are affected by salt stress, and the extent of these soils is constantly expanding due to anthropogenic activities and climate change (Sofy et al. 2021b). Environmental stresses are thought to be responsible for half of all crop losses, posing a severe danger to global food security (Mohamed et al. 2021a). In addition, food production must be boosted by 70% by the end of 2050 as a result of the significant increase in the world population (Agha et al. 2021). Egypt has suffered from a significant dearth of agricultural water in recent days, particularly since the completion of the Grand Ethiopian Renaissance Dam (Sofy et al. 2021c). According to UN forecasts based on recent studies, the world’s population would reach roughly ten billion people by 2050, resulting in a 50% rise in agricultural demand, and considering that agriculture now consumes 69% of freshwater withdrawn globally. As

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Soil salinity limits plant growth and production due to osmotic stress, which is followed by ionic toxicity from the buildup of Na⁺ and Cl⁻ ions in the cell cytoplasm, resulting in oxidative stress and a deficit of nutritional stress (Mohamed et al. 2021b). One of the most harmful salinity impacts is oxidative damage induced by a considerable concentration of reactive oxygen species (ROS) (Saddiq et al. 2021). The accumulation of ROS promotes the oxidation of proteins and lipids (Sofi et al. 2021a). It obstructs normal cellular activity by causing the malfunction of critical cellular organelles, such as chloroplasts (Nigam et al. 2022). Plants have multiple defense immunities to sustain under environmental changes, such as triggering osmolyte formation, which contributes to greater water uptake, the establishment of physiological mechanisms (El-Sheshawy et al. 2021; Naeem et al. 2020), and activation of the antioxidant defense system for rapid removal of ROS (Abu-Shahba et al. 2022; Ghonaim et al. 2021). Non-enzymatic compounds like glutathione (GSH) and ascorbate (ASA) can also successfully scavenge ROS in addition to enzymatic antioxidant mechanisms. Salt stress causes an overabundance of Na⁺ in plant cells, which competes with K⁺ uptake, resulting in a K⁺ deficit (Sofi et al. 2020b). The most effective strategy to minimize Na⁺ buildup in plants is to restrict Na⁺ input into the root in the first place, which will increase crop plant salt tolerance (Jiang et al. 2019).

Some biologically derived compounds found in plants, such as phytohormones and signal molecules, could be considered a significant tool climate (Abd El-Rahman and Ünay 2018) as well as phytohormones and signal molecules, could be considered a significant tool climate (Abd El-Rahman and Ünay 2018) as a result of its ability to react with thiol groups (Oz and Ünay 2021). Furthermore, exogenous H₂S has been shown to boost salt resistance in poplar (Zhao et al. 2018) and cucumber (Jiang et al. 2019).

Phaseolus vulgaris L. is a useful legume crop in Egypt and many other nations (Mohamed et al. 2021b). Furthermore, P. vulgaris provides roughly half of the world’s grain legumes, providing a high amount of protein and nutrients in human nutrition that are critical for human health (Mohamed et al. 2021a). Furthermore, common beans are considered a salt-sensitive crop and suffer from yield reductions in soil containing salinity levels of less than 2 d Sm⁻¹ (Assimakopoulou et al. 2015).

This study aims to assess the effect of different presoaking levels of H₂S in the form of NaHS on improving the tolerance to salinity of common bean plants by (1) examining the influence of H₂S on oxidative stress, osmolytes, endogenous nitric oxide (NO), and the antioxidant system in common bean plants under salt stress and (2) investigating the role of H₂S in ion homeostasis.

2 Material and Methods

2.1 Plant Material and Chemicals

The Agricultural Research Center, Ministry of Agriculture, Giza, Egypt kindly gave common bean seeds (Phaseolus vulgaris L. cv. Nebraska). NaHS (sodium hydrosulfide, Sigma-Aldrich) was used as a hydrogen sulfide donor.

2.2 Experimental Procedures

Seeds were surface washed in a 1% sodium hypochlorite solution for 2 min and then rinsed several times in sterile distilled water. A polyethylene pot (35 cm in diameter) was filled with 6 kg of air-dried clay loam soil, which contained 28.5% sand, 22.1% silt, and 49.4% clay. On average, the temperatures ranged from 17.5 to 35.5 °C, with an average daily relative humidity of 40–81.2%. Table 1 shows the chemical composition of the soil. The seeds were soaked in distilled water at room temperature (act as control plants). The other seeds were soaked in two concentrations of sodium hydrosulfide (NaHS, 50 and 100 µM, a hydrogen sulfide donor) for 8 h and then air drying for 2 h. After air drying, the seeds were sown in pots with approximately six seeds per pot and watered with tap water. After 10 days, the seedlings were thinned to four seedlings per pot. In a completely randomized design (CRD), the pots were divided into nine treatments with four replications as follows: T1 (irrigated with water as control); T2 (50 µM NaHS); T3 (100 µM NaHS); T4 (irrigated with 75 mM NaCl); T5 (50 µM NaHS + 75 mM NaCl); T6 (100 µM NaHS + 75 mM NaCl); T7 (irrigated with 50 mM NaCl); T8 (irrigated with 75 mM NaCl); T9 (irrigated with 100 mM NaCl).
NaCl); T6 (100 μM NaHS + 75 mM NaCl); T7 (irrigated with 150 mM NaCl); T8 (50 μM NaHS + 150 mM NaCl); and T9 (100 μM NaHS + 150 mM NaCl). The irrigation with NaCl solutions of common bean plants began 25 days after seed sowing. Irrigation with tap water was performed once after every three irrigations with NaCl solutions.

2.3 Data Collection

After 60 days of sowing, from each treatment, ten plants were chosen in random form. Six samples from each treatment were used for morphological determinations (plant length, shoots, fresh roots, and dry weight).

2.4 Determination of Photosynthetic Pigments

The Lichtenthaler and Wellburn (1983) method assessed chlorophyll (Chl), carotenoid (Cart) concentration, and total pigments. Half a gram of common bean leaves was ground with acetone at a concentration of 80%, kept overnight, and then filtered with a Whatman filter paper. A spectrophotometer was used to measure the supernatants at wavelengths of 663 and 645 nm (for Chl) and 470 nm (for carotenoids).

2.5 Determination of Osmolytes

Bates et al. (1973) used a ninhydrin reagent to determine the proline content in common bean leaves after being ground in sulfsalicylic acid (3%). First, the wavelength was read at 520 nm using a spectrophotometer. Next, half a gram of fresh common bean leaves was mashed in 5 mL of toluene–water to extract glycine betaine and determined by using 1,2-dichloroethane. Finally, the optical density was read at 365 nm using a spectrophotometer according to the Grieve and Grattan (1983) approach.

2.6 Determination of Malondialdehyde

Heath and Packer (1968) determined the malondialdehyde (MDA) content using 2% thiobarbituric acid after curing half-gram leaf samples in 0.1% (w/v) trichloroacetic acid. The optical density was read at 532 and 600 nm using a spectrophotometer.

2.7 Determination of Electrolyte Leakage

Szalai et al. (1996) protocol was used to measure electrolyte leakage (EL), and fresh leaf discs were boiled in 10 mL of deionized water, and electrical conductivity (EC1) was measured. Subsequently, the tubes were heated for 30 min at 55 °C, and the electrical conductivity (EC2) was measured once more. Finally, the electrical conductivity (EC3) was measured again after boiling the tissue for 10 min at 100 °C. Again, the formula was used in the calculation:

Electrolyte leakage (%) = \(\frac{EC2 - EC1}{EC3} \times 100\)

2.8 Determination of ROS Indicators

Velikova et al. (2000) used TiCl2 to measure \(H_2O_2\) hydrogen peroxide after extracting half a gram of common bean leaves in \(KH_2PO_4-KOH\) buffer (pH 7.8). The optical density was read at 410 nm using a spectrophotometer.

Babbs et al. (1989) estimated the concentration of hydroxyl radicals (\(\cdot OH\)). The reaction mixture contained deoxyribose, \(KH_2PO_4-KOH\) buffer (20 mM, pH 7.4), 100 μM of FeCl3, 104 μM of EDTA, 1 mM \(H_2O_2\), and 100 μM of ascorbate in the final after incubation at 37 °C for 1 h. The absorbance was read at 532 nm using a spectrophotometer.

The superoxide anion production rate (\(O_2 \bullet-\)) was measured according to the method of Green and Hill (1984). A gram of fresh plant tissue was ground with 50 mM phosphate buffer, 2% PVP-30, and 0.5% Triton X-100, and the optical density of the mixture was read at 530 nm using a spectrophotometer. The oxygen output rate was determined on the standard NaNO2 linear curve.

2.9 Determination of Relative Water Content

The relative water content (RWC) was measured using the formula provided by Henson et al. (1981)

\[ RWC = 100 \times \frac{\text{fresh mass} - \text{dry mass}}{\text{turgid mass} - \text{dry mass}} \]

Turgid mass was calculated after leaf blades were saturated in distilled water in covered glass jars for 4 h at room temperature and then kept overnight at 5 °C. Subsequently,
the samples were kept for 48 h at 80 °C, and then the dry mass was weighed.

2.10 Determination of Hydrogen Sulfide

Li et al. (2012) method was used to determine hydrogen sulfide after extracting the common bean leaves in KH₂PO₄ (pH 7) and using 5,5′-dithiobis (2-nitrobenzoic acid) reagent. The optical density was read at 412 nm using a spectrophotometer.

2.11 Determination of Nitric Oxide

Zhou et al. (2005) protocol was used to measure NO content in common bean leaves after being ground in acetic acid buffer (50 mM, pH 3.6) and using Griess reagent (1.0% sulfanilamide and 0.1%-1-naphthyl ethylenediamine dihydrochloride in a 0.5% H₂PO₄ solution). The wavelength of the solution was read at 540 nm using a spectrophotometer.

2.12 Determination of Non-enzymatic Antioxidants

Mukherjee and Choudhuri (1983) method measured ascorbate in common bean leaves crushed in liquid nitrogen, and 2% dinitrophenylhydrazine, 6% trichloroacetic acid, and 10% thiourea were used to determine ascorbate. The optical density was read at 530 nm using a spectrophotometer. The Griffith (1980) technique measured total glutathione (GSH) in common bean leaves after being ground in 4% sulfosalicylic acid and using Ellman’s reagent to assay GSH. The optical density was read at 412 nm using a spectrophotometer.

2.13 Determination of Enzymatic Antioxidants

One gram of fresh leaves of common bean plants was crushed with 0.05 M phosphate buffer (pH 7.0) at 4 °C. The mixture was centrifuged at 10,000 × g for 20 min at 4 °C. For determination, the supernatant was brought to a known volume and kept in a refrigerator. Marklund and Marklund (1974) method was used to assay superoxide dismutase activity (SOD, EC 1.15.1.1), Chen et al. (2000) methods were used to measure catalase activity (CAT, EC1.11.1.6), Hammerschmidt et al. (1982) method was used to assay peroxidase activity (POX, EC 1.11.1.7), Jiang and Zhang (2002) methods were used to determining glutathione reductase activity (GR, EC 1.8.1.6), and Nakano and Asada (1981) methods were used to measure ascorbate peroxidase (APX, EC 1.11.1.11). Nitrate reductase activity (NR, EC 1.7.1.1) was measured spectrophotometrically as described (Chaki et al. 2011).

2.14 Determination of Mineral Content

Five grams from dry leaves of common bean plants was digested using nitric acid and sulfuric acid. The digestion solution yielded estimates of nitrogen (N), phosphorus (P), and potassium (K). The micro-Kjeldahl approach and the flame photometric method determined total N (Velikova et al. 2000) and K (Sen Tran et al. 1988), respectively. P was determined using a molybdenum reagent. In addition, Na⁺ was separated for 3 h in boiling water and then measured using a flame photometer (Wolf 1982).

2.15 RNA Isolation, cDNA Synthesis, and Quantitative Real-time PCR Analysis

The TRIzol technique was used to isolate total RNA. The Bioneer Exicycler Tm 96 FaST instrument performed quantitative real-time PCR (qRT–PCR). Table 2 lists the primer sequences utilized in the research. The control primer was based on the actin housekeeping gene actin. For PCR amplification, a list of genes and primers was created. SYBR Green I dye was used to evaluate PCR product amplification (Chang et al. 1993).

2.16 Statistical Analysis

The SPSS software package version 28.0 (SPSS, Chicago, USA) was used to analyze variance (two-way ANOVA) according to Duncan’s multiple range test at a significance level of 5%. The heatmap shows the examination of the Pearson correlation. Additionally, it was calculated to see if there

| Primer sequence (5′–3′) |
|-------------------------|
| **Reactive oxygen species (ROS)** |
| Copper-zinc superoxide dismutase (Cu-ZnSOD) F: CAAGAGTTCACTGGTGAACC CACTGCA R: TCCAGAAAAACAG |
| Catalase (CAT) F: CACATCCAGGAAATGGAAGG R: CCAGCTTTTGCATGAGGGG |
| Glutathione S-transferase (GST) F: AGCTCTCAGGAGACACTGAGCAA R: AGGAGGTGGTAAGAGG |
| Actin F: TGACTACGTTGGTGATGAGG R: AGGAGGTGGTAAGAGG |
was a link between quantitative factors. The graphs were created using GraphPad Prism 8.

3 Results

3.1 Changes in Morphological Criteria

Under normal conditions, the application of 50 and 100 µM NaHS caused significant enhancement in the morphological criteria like plant height of about 5.92% and 14.58%, shoot fresh weight of about 8.57% and 15.72%, root fresh weight of about 43.84% and 73.84%, and root dry weight of about 25.00% and 62.50%, respectively, over control plants (Fig. 1a-e). However, irrigation of common bean plants with various levels of salt stress (75 and 150 mM NaCl) caused a significant decrease in plant height by 35.40% and 45.15%, shoot fresh weight of approximately 34.29% and 50.00%, shoot dry weight of approximately 31.94% and 50.19%, root fresh weight of about 50.77% and 62.31%, and root dry weight of approximately 33.89% and 65.70%, respectively, compared to non-stressed plants. On the other hand, treatment with 50 and 100 µM NaHS caused a significant boost in all morphological criteria compared to salt-stressed plants (75 and 150 mM).

3.2 Changes in Photosynthetic Pigments

Significant reductions were obtained in chlorophyll-a (33.3%, 47.4%), chlorophyll-b (45.6%, 66.2%), carotenoids (37.2%, 61.5%), and total photosynthetic pigment content (37.9%, 55.8%) of common bean plants irrigated with 75 and 150 mM NaCl, respectively, compared to untreated plants. On the other hand, the application of 50 and 100 µM NaHS to non-salinized plants caused a significant increase in Chl a (116.7%, 33.3%), Chl b (9.9%, 67.3%), carotenoids (21.2%, 35.3%), and total pigment content (15.3%, 44.4%), respectively, compared to control plants (Fig. 2a-d). Furthermore, the photosynthetic parameters were improved for salinized plants with NaHS treatment. Compared to salt-stressed plants, treatment with 50 and 100 µM NaHS resulted in a considerable boost in photosynthetic pigment content. In this regard, higher values of chlorophyll-a (66.9%), chlorophyll-b (140.0%), carotenoids (61.6%), and total pigment content (58.1%) were detected in plants irrigated with 150 mM NaCl and treated with 100 µM NaHS compared to those irrigated with 150 mM NaCl alone (Fig. 2a-d).

3.3 Changes in Osmoprotectants (Proline and Glycine Betaine)

Higher values of proline (37.8%, 91.1%) and glycine betaine (GB) (80.0%, 91.1%) were detected in plants irrigated with different concentrations of salt stress (75 and 150 mM), respectively, when compared to untreated plants (Fig. 3a-b). Furthermore, treating non-salinized common bean plants with 50 and 100 µM NaHS increased proline (18.5%, 9.6%) and glycine betaine (16.8%, 35.8%), respectively as compared to control plants. Furthermore, treatment of salt-stressed plants with 50 and 100 µM NaHS increased proline and GB content compared to salt-stressed plants alone (Fig. 3a-b). Common bean plants treated with 100 µM NaHS and irrigated with 75 and 150 mM exhibited higher values in proline (22.6%, 14.7%) and GB (25.1%, 18.5%), respectively.

3.3.1 Changes in Oxidative Stress and Relative Water Content

Irrigation of common bean plants with 75 and 150 mM NaCl caused increments in MDA (60.6%, 104.2%), EL (80.1%, 130.7%), H2O2 (67.2%, 102.5%), O2- (58.9%, 78.5%), and OH (62.9%, 85.3%) respectively as compared to control plants (Figs. 3, 4), while RWC decreased by 15.5% and 35.4%, respectively, compared to the control plants (Fig. 3e). In addition, in comparison to untreated plants, treatment of common bean plants with 50 and 100 µM NaHS resulted in a considerable reduction in MDA, EL, H2O2, O2-, and OH contents as well as a significant increase in RWC (Figs. 3, 4). Furthermore, the application of 100 µM NaHS caused a reduction in MDA (21.9%, 17.9%), EL (16.0%, 20.7%), H2O2 (13.25%, 15.8%), O2- (16.2%, 15.1%), and OH (13.7%, 13.2%) but caused an increase in RWC (13.0%, 28.2%) contents in salt-stressed plants irrigated with 75 and 150 mM NaCl, respectively (Figs. 3, 4). Furthermore, NaHS treatment further reduced the oxidative stress parameters, showing that the external application of NaHS had a positive effect on reversing the oxidative stress and reducing cell membrane injury.

3.3.2 Changes in Endogenous H2S and Nitric Oxide Content

Common bean plants irrigated with 75 and 150 mM NaCl showed an increment of endogenous H2S content of 23.2% and 10.6%, respectively as compared to control plants. On the contrary, the application of 50 and 100 µM NaHS treatment increased the endogenous H2S content by 6.3% and 10.2%, respectively, compared to the control plants (Fig. 4c-d). Furthermore, the addition of 100 µM NaHS to plants irrigated with 75 and 150 mM salt stress...
boosted H$_2$S levels by 16.8% and 53.0%, respectively, as compared to salt-stressed plants alone. Moreover, treatment with 75 and 150 mM NaCl significantly elevated the endogenous NO levels by approximately 65.4% and 156.4%, respectively, compared to control plants (Fig. 4c-d). Furthermore, treatment with 100 µM NaHS enhanced the overproduction of NO content by 26.3% and 24.0%, respectively, in plants stressed with 75 and 150 mM salt.
Effect of different concentrations of NaHS on common beans grown under different levels of salt stress. The values are represented as means ± SE (n = 6), each bar followed by a lower-case letter. Different letters next to the mean values in each column indicate significant differences. In the two-way analysis of variance (ANOVA), there are significant and very significant differences indicated by ** and *** respectively. T1 (irrigated with water as control); T2 (50 µM NaHS); T3 (100 µM NaHS); T4 (irrigated with 75 mM NaCl); T5 (50 µM NaHS + 75 mM NaCl); T6 (100 µM NaHS + 75 mM NaCl); T7 (irrigated with 150 mM NaCl); T8 (50 µM NaHS + 150 mM NaCl); and T9 (100 µM NaHS + 150 mM NaCl).

### 3.4 Changes in Non-enzymatic Antioxidants

Compared to untreated plants, significant increases in ASA (31.17%, 62.34%) and GSH (14.17%, 62.73%) were observed in plants irrigated with 75 and 150 mM NaCl, respectively (Fig. 4e-f). However, salt-stressed plants supplemented with 50 and 100 µM NaHS exhibited higher concentrations of ASA and GSH levels than both non-stressed and salt-stressed plants. The most pronounced increases in ASA content (15.8%, 10.0%) and GSH (25.3%, 23.4%) were detected in common bean plants irrigated with 75 and 150 mM NaCl, respectively, supplemented with 100 µM NaHS.

### 3.5 Changes in Antioxidant Enzymes

The activities of antioxidant enzymes (CAT, POX, SOD, APX, GR, NR) in leaves of common bean plants were measured to assess the effect of H2S in modulating salt stress (Fig. 5a-f). In comparison to control plants, the activity of CAT, POX, SOD, APX, GR, and NR significantly increased with increasing NaCl concentrations. Higher activities of CAT (65.3%), POX (43.4%), SOD (134.8%), APX (140.4%), GR (43.4%), and NR (20.5%) were detected in plants irrigated with high concentrations of salt stress (150 mM) as compared to unstressed plants. In addition, treatment with 100 µM NaHS caused a significant increase in CAT (23.5%), POX (17.1%), SOD (44.9%), APX (45.4%), GR (23.1%), and NR (28.3%) as compared to control plants (Fig. 5). Furthermore, treatment with 100 µM NaHS alleviated the adverse effect of high concentrations of NaCl (150 mM) by upregulating antioxidant activity and reported higher values in CAT (14.2%), POX (16.8%), SOD (14.2%), APX (25.9%), GR (14.9%), and NR (7.5%) over those of the corresponding salt-stressed levels alone.

### 3.6 Changes in Mineral Concentration

Maintaining a lower Na+/K+ ratio in plants is essential for their capacity to endure salt stress. Compared to control plants, plants irrigated with 75 and 150 mM salt stress showed a significant increase in Na+ (133.5%, 361%) and Na+/K+ content (254.4%, 720%), respectively. However, common bean plants irrigated with 75 and 150 mM showed a substantial decrease in N (38.5%, 59%), P (37.4%, 56.8%), and K+ (49.4%, 56.8%) contents respectively as compared to untreated plants (Fig. 6).

In addition, compared to salt-stressed plants, treatment with 50 and 100 µM NaHS showed a significant increase in N, P, and K+ concentrations while a significant decrease in Na+ content in the leaves of common bean plants. The most pronounced increases in N (45.8%, 50%), P (43.9%, 55%), and K+ (85.4%, 68.6%) were detected in plants treated with 100 µM NaHS and irrigated with 75 and 150 mM NaCl, respectively, relative to salt-stressed plants alone. Furthermore, compared to plants treated with NaCl alone, the Na+/K+ ratio of NaHS treated plants was considerably lower (Fig. 6). Under salt stress, H2S reduces excessive Na+ absorption and preserves the Na+/K+ balance at the cellular level. These findings suggest that externally administered NaHS is more successful in increasing K+ concentration while decreasing Na+ content and so increasing salt stress tolerance in common bean plants (Fig. 6).

### 3.7 Changes in Gene Expression

Significant increases in Cu-ZnSOD, CAT, and GST relative expression levels were observed in salinity stress common plants treated with NaHS compared to salt-stressed plants (Fig. 7). In this regard, seeds soaking with 100 µM NaHS showed a significant upregulation of Cu-ZnSOD, CAT, and GST relative expression levels by about 59.57%, 46.67%, and 71.42% when irrigated with 75 mM NaCl and 36.47%, 69.64%, and 50.69% when irrigated with 150 mM NaCl, respectively, compared to salt-stressed plants without any treatments (Fig. 7).

### 3.8 Heatmap Correlation

The results of the correlation analysis under H2S and salinity showed that the height of the plant, the fresh weight (FW) of the shoot, the dry weight (DW) of the shoot, the root of the FW, the root of the DW, the photosynthetic pigments (Chl a, Chl b, Cart, total pigments), proline, GB, MDA, EL, H2O2, H2S, NO, RWC, ASA, GSH, SOD, POX, APX, CAT, GR, N, P, K, Na, and Na+/K+ were significantly correlated (Fig. 8). There were positive and significant correlations among morphological characteristics and photosynthetic pigments, H2S, RWC, N, P, and K+. On the contrary, there were negative and significant correlations between morphological characteristics and proline, GB, MDA, EL, H2O2, NO, ASA, GSH, SOD, POX, APX, CAT, GR, Na, and Na+/K+ (Fig. 8).
4 Discussion

The data in the present study showed that salinity stress decreased plant growth by decreasing plant height, FW of shoots and roots, and DW of shoots and roots of common bean plants compared with control plants. One of the most negative impacts of salt stress is the reduction in plant growth (Naeem et al. 2020). The reduced plant growth due to salt stress could be due to low uptake of essential nutrient elements (Dawood et al. 2021), calcium (Methenni et al. 2018), and Na⁺ toxicity (Mohamed et al. 2021b). The decline in plant growth caused by salt stress could also be attributed to the decline in cell division and elongation (Arif et al. 2020). Similar results are reported by Gandonou et al. (2018), who found that salinity stress inhibited plant growth and K⁺ content of leaves and caused a high accumulation of Na⁺ concentration.

Reduced water absorption in plants as a result of osmotic stress caused by salinity is another possible explanation for lower plant growth (Gandonou et al. 2018). This could be due to the destructive impact of NaCl salinity on the stomatal impedance to water flow and hormonal imbalances (Arif et al. 2020).

NaHS, as an H₂S donor, considerably reduced the inhibition of morphological characteristics caused by salt stress. Similarly, the reversion of salt stress responses by H₂S has been observed in some plant species such as *Oryza sativa* (Mostofa et al. 2015), *Nicotiana tabacum* (Da Silva et al. 2017), and cauliflower (Ahmad et al. 2020). Our results are similar to Mostofa et al. (2015), who found that H₂S increased plant growth and biomass of salt-stressed rice which could be attributed to its role in protecting Chl a, Chl b, carotenoids, and proteins from salt-induced damage. Furthermore, treatment of cucumber

![Fig. 2 Effect of different concentrations of NaHS on plant growth parameters under different levels of salt stress.](image-url)
Fig. 3 Effect of different concentrations of NaHS on a proline, b glycine betaine, c malondialdehyde (MDA), d electrolyte leakage, e relative water content (RWC), and f hydrogen peroxide (H$_2$O$_2$) in common bean leaves under different levels of salt stress. The values are represented as means ± SE (n = 3), each bar followed by a lower-case letter. Different letters next to the mean values in each column indicate significant difference. In the two-way analysis of variance (ANOVA), there are significant and very significant differences indicated by ***, respectively, ns indicate non-significant. T1 (irrigated with water as control); T2 (50 µM NaHS); T3 (100 µM NaHS); T4 (irrigated with 75 mM NaCl); T5 (50 µM NaHS + 75 mM NaCl); T6 (100 µM NaHS + 75 mM NaCl); T7 (irrigated with 150 mM NaCl); T8 (50 µM NaHS + 150 mM NaCl); and T9 (100 µM NaHS + 150 mM NaCl)
Fig. 4 Effect of different concentrations of NaHS on a superoxide anion (O$_2^-$), b hydroxyl radicals (OH), c hydrogen sulfide (H$_2$S), d nitric oxide (NO), e ascorbic acid (ASA), and f glutathione (GSH) in common bean leaves under different levels of salt stress. The values are represented as means ± SE ($n = 3$), each bar followed by a lowercase letter. Different letters next to the mean values in each column indicate significant difference. In the two-way analysis of variance (ANOVA), there are significant and very significant differences indicated by *, **, and ***, respectively. T1 (irrigated with water as control); T2 (50 µM NaHS); T3 (100 µM NaHS); T4 (irrigated with 75 mM NaCl); T5 (50 µM NaHS + 75 mM NaCl); T6 (100 µM NaHS + 75 mM NaCl); T7 (irrigated with 150 mM NaCl); T8 (50 µM NaHS + 150 mM NaCl); and T9 (100 µM NaHS + 150 mM NaCl)
seeds with NaHS under salinity stress caused induction of the germination of seeds, which may be related to the activation of starch hydrolysis in the endosperm due to the enhancement in the activity of α-amylase and β-amylase, resulting in stimulation of plant growth (Sun and Luo 2014).
Fig. 6 Effect of different concentrations of NaHS on mineral content (a nitrogen (N), b phosphorus (P), c potassium (K), d sodium (Na), and e sodium/potassium (Na/K)) in common bean leaves under different levels of salt stress. The values are represented as means ± SE (n = 3), each bar followed by a lower-case letter. Different letters next to the mean values in each column indicate significant difference. In the two-way analysis of variance (ANOVA), there are significant and very significant differences indicated by ***, respectively. T1 (irrigated with water as control); T2 (50 µM NaHS); T3 (100 µM NaHS); T4 (irrigated with 75 mM NaCl); T5 (50 µM NaHS + 75 mM NaCl); T6 (100 µM NaHS + 75 mM NaCl); T7 (irrigated with 150 mM NaCl); T8 (50 µM NaHS + 150 mM NaCl); and T9 (100 µM NaHS + 150 mM NaCl).
Salinity has a particularly negative impact on photosynthesis and other metabolic activities (Sofy et al. 2021c). In our results, the levels of Chl (a) and Chl (b), carotenoids, and total photosynthetic pigment contents in common bean leaves decreased substantially under salt stress. The decrease in chlorophyll in common bean plants could be related to oxidative damage caused by salt stress (Mohamed and Abd-El Hameed 2014; Taïbi et al. 2016). Furthermore, the chlorophyllase enzyme might have been involved in blocking or deprivation of chlorophyll synthesis and stimulation of chlorophyll degradation (Dawood and Azooz 2020; Fouda and Sofy 2022). Furthermore, our results showed that H₂S alleviated the reduction in the photosynthetic parameters. Our results are consistent with those of Mostofa et al. (2015), who found that H₂S increased overall growth and productivity in salt-stressed rice plants, possibly due to its function in preserving Chl a and Chl b from salt-induced damage. On the other hand, this considerably restricted the decrease in chlorophyll concentration, which could be attributed to the prevention of ROS formation or the stability of the maintenance of the photosynthetic mechanism (Jiang et al. 2019; Younes et al. 2020). Furthermore, H₂S may have contributed to improving photosynthetic performance and thus the overall performance of rice plants by delivering Mg, Fe, Zn, and Mn ions as well as by promoting chloroplast biogenesis and increasing the ability of CO₂ fixation, as observed in Spinacea oleracea (Chen et al. 2011) and cucumber plants (Jiang et al. 2019), and increased photosynthetic pigments by protecting cell membrane organelle containing chlorophyll against salinity-induced ion toxicity (Agha et al. 2021).

Salinity stress and H₂S treatment caused a significant increase in osmolytes in the leaves of common bean plants. Similar to our results, treating pepper plants with 100 mM NaCl increases the accumulation of proline and glycine betaine that triggers the accumulation of osmolytes for better protection of cellular functioning (Kaya et al. 2020). Furthermore, Chang et al. (2014) found that proline synthesis in Catharanthus roseus was inversely related to RWC, biomass, and K⁺ buildup under salt stress conditions. Under salt stress, treatment with H₂S increased RWC in strawberry leaves (Christou et al. 2013) and proline in rice (Mostofa et al. 2015). Furthermore, H₂S treatment plays an important role: (1) maintaining relative water content by enhancing proline and soluble sugar buildup to maintain osmotic potential below the ionic medium (Kaya et al. 2020); (2) decreasing the hydraulic conductivity, possibly as a result of a drop in the rate of water channels (aquaporins) (Bashandy et al. 2020; Kaldenhoff et al. 2008); and (3) improving the expression of proteins associated with the manufacture of osmolytes, primarily proline, and being linked to a drop in enzymes that catabolize it (Jiang et al. 2019; Sallam et al. 2021). In addition, glycine betaine is an osmoprotection that plays a role in upregulating enzyme activity and, as a result,
maintaining membrane structure and fluidity against the damaging effects of salt stress (Sofy et al. 2020a).

Plants suffering from NaCl toxicity often exhibit symptoms associated with oxidative stress and membrane lipid peroxidation, which can result in the accumulation of ROS and MDA (Abdelrhim et al. 2021). Salinity stress causes an accumulation of oxidative stress markers such as \( \text{H}_2\text{O}_2 \), MDA, and EL (Kaya et al. 2020). In addition, high salinity-triggered overproduction of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) in leaf tissues was correlated with the significant increase in MDA level in rice plants (Mostofa et al. 2015). In this study, high salinity triggered overproduction of \( \text{H}_2\text{O}_2 \), increased electrolyte leakage, and caused accumulation of MDA in common bean leaves, whereas caused a reduction in RWC content. \( \text{H}_2\text{O}_2 \) alleviated salinity-induced oxidative damage, as evidenced by reduced \( \text{H}_2\text{O}_2 \), MDA, and electrolyte leakage levels in common leaves and increased RWC. Under salt stress, considerable amounts of free radicals were formed in \textit{Cyclocarya palicur}us, leading to membrane peroxidation through an increase in MDA, which impaired cell equilibrium and greatly hindered plant growth, according to Chen et al. (2021). Plants produce antioxidant enzymes to keep ROS levels constant, and when ROS levels rise dramatically under stress, the antioxidant enzyme system is triggered. Furthermore, \( \text{H}_2\text{S} \) mitigated salt stress by lowering \( \text{H}_2\text{O}_2 \), MDA, and electrolyte leakage in common bean leaves while also increasing RWC. Similar studies found that \( \text{H}_2\text{S} \) decreased salt stress by lowering \( \text{H}_2\text{O}_2 \), \( \text{O}_2 \), and MDA levels and generating reactive oxygen species through an increased antioxidant system in rice (Mostofa et al. 2015) and cucumber seedlings (Jiang et al. 2019).

One more crucial mechanism operative in plants is an adaptation to salinity stress by regulating the antioxidant defense system by inducing ascorbate (ASA) and glutathione (GSH), which are important for eliminating ROS and maintaining cellular redox potential (Younes et al. 2021), as shown in our results. In our study, the contents of ASA and GSH were elevated in response to the formation of \( \text{H}_2\text{O}_2 \) to counteract oxidative damage in salinity-stressed plants. NaHS treatment improved the content of ASA and GSH, as well as CAT, SOD, APX, GR, POX, and NR activities in common bean leaves under NaCl stress. Jiang et al. (2019) observed that NaHS treatment significantly enhanced ASA and GSH concentrations as well as SOD, GR, and POX activities in cucumber leaves under salt stress. Furthermore, Mostofa et al. (2015) showed that \( \text{H}_2\text{S} \) performed its antioxidant function in salt-stressed rice plants by boosting the levels of ASA and GSH and restoring redox homeostasis. Moreover, enhancement of SOD, CAT, and POX activities also scavenged ROS by converting superoxide \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \) at the first stage of the defense system, and indicated that \( \text{H}_2\text{S} \) kept balance between ROS production and detoxification thereby protecting cells from oxidative damage. Furthermore, \( \text{H}_2\text{S} \) increased the activity of APX and GR, which work together to eliminate \( \text{H}_2\text{O}_2 \) and produce ASA and GSH, which are important for improving plant resistance to salt stress (Maksoud et al. 2022; Mostofa et al. 2015). Furthermore, a careful analysis of the defense mechanism
under H2S addition revealed that H2S further improved the level of GSH, possibly by inducing its biosynthesis or by upregulating GR activity under salt stress, which then participates in redox homeostasis (Mostofa et al. 2015). NR is a key enzyme responsible for endogenous NO biosynthesis in plants (Liang et al. 2018). NR-mediated NO production is involved in salt stress (Khan et al. 2012).

Salinity stress and NaHS caused a significant boost in endogenous H2S and NO in common bean leaves. A similar study showed that salt stress causes a significant increase in endogenous H2S formation in some plant species, like strawberries (Christou et al. 2013) and alfalfa (Lai et al. 2014). The activation of the endogenous H2S level after stress treatments indicated that H2S could also be an important secondary messenger of stress detection, which in turn modulated plant physiological changes and downstream gene expressions (Christou et al. 2013). In addition to amplifying the H2S signal, the significant increase in endogenous H2S content will further regulate dynamic S metabolism in plants, thus promoting the production of sulfur derivatives (i.e., cysteine and GSH) and sulfur-containing proteins (Li et al. 2020). Furthermore, H2S improves salt resistance in barley seedlings via NO-mediated ion homeostasis (Chen et al. 2015). Furthermore, the level of NO and nitrate reductase (NR) activity, the main enzyme for NO production, was significantly boosted by salt and H2S treatment (Ma et al. 2019). In addition, exposure of Cyclocarya paliurus to exogenous H2S induced endogenous NO production, which contributed to plant salt resistance (Chen et al. 2021).

To maintain their structure and vital physiological activities, plants require sufficient amounts of nutrients (Mowafy et al. 2022). When such nutrients are insufficient, metabolic processes may be disrupted (Gomaa and Dawood 2021). Because of the antagonistic impact of Na+ on K+ binding sites, salinity causes excessive Na+ buildup due to potassium (K+) insufficiency (Gul et al. 2016). Plant salinity resistance is correlated with their optimal K+/Na+ ratio (Hanin et al. 2016). Salinity stress reduces the K+/Na+ ratio due to reducing K+ levels in plant leaves compared to Na+ (Assaha et al. 2017). Plants need to maintain their homeostasis of N, P, K+, and Na+ ions to survive under salt stress. Our results showed that H2S hindered Na+ uptake and promoted N, P, and K+ uptake in common bean leaves. Increases in cations and their salts in the soil generate an external osmotic potential that can prevent or reduce the influx of water into the root. The resulting water deficit is similar to drought conditions and additionally compounded by the presence of Na+ ions (Ekinci et al. 2021). Treatment with H2S increased salinity by decreasing Na+ and boosting K+ and the K+/Na+ ratio in many plants, for example, alfalfa (Lai et al. 2014), rice (Mostofa et al. 2015), and cauliflower (Ahmad et al. 2020). Treatment with H2S has been reported to lower the intracellular Na+ concentration and the Na+/K+ ratio and block intracellular K+ exosmosis in various plants, like rice and Malus hupehensis (Li et al. 2020; Mostofa et al. 2015).

Based on the action of the expression of the housekeeping gene, changes in the expression levels of Cu/Zn SOD, GST, and CAT expression levels, known as antioxidant stress genes, were determined. The link between these levels of expression and H2O2 has been established. Our results showed that Cu/Zn SOD, GST, and CAT gene expression levels were maximum at 100 µM NaHS. On H2O2 other hand, the concentrations increased steadily. The continuous increase in H2O2 and the decrease in maximum Cu/Zn SOD, GST, and CAT expression levels at 100 µM NaHS support studies emphasizing H2O2 as a signaling molecule (Farnese et al. 2016). Similar results suggested that the protective impact of H2S could be attributed to improved antioxidant enzyme activity, which could be partially attributed to the elevation of antioxidant enzyme gene expression and, hence, lower MDA buildup (Wang et al. 2012).

5 Conclusion

We believe that hydrogen sulfide (H2S) treatment could be a useful method to improve the growth of common bean plant growth under salt stress. For the following reasons, first, H2S prevents sodium (Na+) from being taken up, hence keeping the (sodium/potassium) Na+/K+ ratio constant. Second, H2S helps maintain mineral homeostasis, which helps enhance physiological and biochemical processes. Third, preserving the photosynthetic apparatus enhances the synthesis of photosynthetic pigments. Fourth, H2S helps prevent oxidative damage and decrease the production of free radicals by regulating antioxidant defenses and the production of glutathione (GSH) and ascorbic acid (ASA). Fifth, H2S boosted the accumulation of endogenous nitric oxide (NO) and H2S, reducing salt-induced oxidative damage and increasing antioxidant enzyme activity, and importantly preventing osmotic stress by overproduction of osmolytes such as proline and glycine betaine. In conclusion, our findings help improve common bean by increasing resistance to salt stress and the production of endogenous H2S and NO levels by applying lower concentrations of H2S.

Author Contribution Mahmoud Sofy: conceptualization, methodology, data curation; Mahmoud Sofy, Ahmed Sofy, Heba Mohamed: investigation, software, validation, formal analysis; Heba Mohamed, Mahmoud Sofy, Mona Dawood, Ahmed Sofy, Huwida Abdelkader: writing—review and editing.

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Declarations

Conflict of Interest The authors declare no competing interests.

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