Chemosphere 309 (2022) 136678

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

The involvement of hydrogen sulphide in melatonin-induced tolerance to arsenic toxicity in pepper (*Capsicum annuum* L.) plants by regulating sequestration and subcellular distribution of arsenic, and antioxidant defense system

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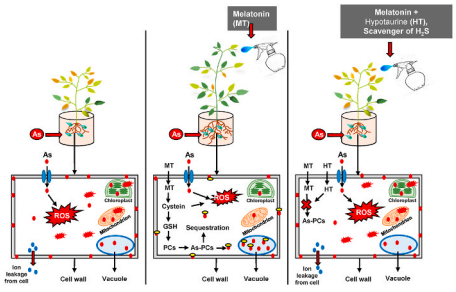
HIGHLIGHTS

- Arsenic stress (AsS) led to a marked reduction in plant growth and oxidative stress in pepper.
- MT markedly eliminated the As-induced growth inhibition and oxidative stress.
- MT increased the intrinsic level of H_2S as well as induced the antioxidant defence system.
- MT promoted accumulation of soluble form of As in root and leaf vacuoles thereby reducing its toxicity.
- The scavenger of H_2S, HT, inverted the effect of MT by decreasing H_2S content.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: T Cutright

Keywords:
Arsenic toxicity
Melatonin
Pepper
Hydrogen sulphide
Antioxidant system

ABSTRACT

Melatonin (MT) and hydrogen sulphide (H_2S) are recognised as vital biomolecules actively taking part in plant defence systems as free radical scavengers and antioxidants against a myriad of biotic and abiotic stressors. However, it has been yet unknown in plants subjected to arsenic (As) toxicity whether or not H_2S interacts with MT to regulate endogenous antioxidant defence system. Prior to beginning As stress (As–S) treatments, MT (0.10 mM) was applied externally to plants daily for three days. AsS was then started for two weeks with As(V) (0.1 mM as $Na_2HAsO_4{\cdot}7H_2O$). The treatment of As reduced plant biomass (24.4%) and chlorophyll *a* (51.7%), chlorophyll *b* (25.9%), while it increased subcellular As in roots and leaves, levels of glutathione (GSH), hydrogen peroxide (H_2O_2), malondialdehyde (MDA), methylglyoxal (MG), H_2S and phytochelatins (PCs) in

Abbreviations: H2S, Hydrogen sulphide; As, Arsenic; GSH, Glutathione; H_2O2, Hydrogen peroxide; MDA, Malondialdehyde; MG, **M**ethylglyoxal; PCs, Phytochelatins; NaHS, Sodium hydrosulphide; $Na_2HAs_4O \bullet 7H_2O$, Sodium hydrogen arsenate heptahydrate; HT, Hypotaurine; Gly I, Glyoxalase I; Gly II, Glyoxalase II; AsA, Ascorbate; DHA, Dehydroascorbate.

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https://doi.org/10.1016/j.chemosphere.2022.136678
Received 23 August 2022; Received in revised form 18 September 2022; Accepted 28 September 2022
Available online 1 October 2022

pepper plants. In As-stressed pepper plants, the application of MT increased plant biomass (16.3%), chlorophyll *a* (52.7%), chlorophyll *b* (28.2%), antioxidant enzymes' activities, and H_2S accumulation, while it lowered the concentrations of MDA and H_2O_2. In As-treated plants, GSH and phytochelatins (PCs) were increased by MT by regulating As sequestration to make it harmless. The addition of MT increased As accumulation in the vacuoles of roots and caused the soluble fraction of As in vacuoles to become less toxic to vital organelles. MT-induced tolerance to As stress was further enhanced using NaHS, a source of H_2S. Hypotaurine (0.1 mM HT), a H_2S scavenger, was applied to the control and As-stressed plants together with MT and MT + NaHS to determine whether H_2S was implicated in MT-induced increased As–S tolerance. By reducing H_2S generation in pepper plants, HT counteracted the beneficial effects of MT, whereas the addition of NaHS to MT + HT restored the negative effects of HT, proving that H_2S is necessary for the pepper plants As-stress tolerance caused by MT.

1. Introduction

There are several different ways that people might become exposed to arsenic, including through water supply, as well as the soil, water, and plant system (Briffa et al., 2020). Arsenic may be absorbed by plants through their roots from irrigation water and the soil, and so it can quickly access to the food chain (Yanez et al., 2018). Excessive amounts of As in irrigation water or soil markedly impair plant growth and yield (Malakar et al., 2019; Tuan and Van Chuong, 2021); these sources can also add a significant amount of arsenic to the diet, thereby putting human health at high risk (Fatoki and Badmus, 2022). In response to high amounts of arsenic, plants produce an excessive quantity of reactive oxygen species (ROS), which might result in the oxidation and malfunctioning of vital macro- and micro-molecules in plant cells (Bali and Sidhu, 2021; Nahar et al., 2022). When generated, an intricate network of defensive mechanisms, notably non-enzymatic and/or enzymatic antioxidants, may reduce the effects of oxidative stress. In order to safeguard the cells from oxidative harm, these defence metabolites enhance the capacity of plants to combat the overabundance of ROS (Ren et al., 2021; Pardo-Hernández et al., 2021). Besides the ascorbate-glutathione cycle, enzymatic defense mechanisms comprise mainly superoxide dismutases (SOD), peroxidases (POD) and catalase (CAT) (Rajput et al., 2021; Qamer et al., 2021). Non-enzymatic antioxidants, such as ascorbate, and glutathione substances can also play the protective function (Mogazy and Hanafy, 2022). Additionally, it has been noted that As stress tolerance is mediated by enzymes connected to the AsA-GSH cycle (Kaya, 2021). However, such a defensive strategy does not consistently operate in numerous plant species, such as pepper. The pepper plant is widely known to be extremely sensitive to As as high concentrations of AS in growth medium have been documented to be quite harmful to the plant (Kaya et al., 2022).

Arsenate can be detoxified by plants by being converted to arsenite and sequestered in vacuoles, via combounds like glutathione (GSH), phytochelatins (PCs) and thiols, which are regarded as the fundamental ligands of different metalloids and metals (Abbas et al., 2018). Such compounds contribute to tolerance of numerous plants to As stress (Shri et al., 2019; Jiang et al., 2022).

Under stressful situations, methylglyoxal (MG), which may harm cell membranes by dissolving proteins and lipids (Majláth et al., 2022), can also be produced by plants (Li, 2022). To get rid of MG, plants promote the activities of key enzymes connected to the glyoxalase system comprising glyoxalase I (Gly I) and glyoxalase II (Gly II) (Garai et al., 2021; Sahoo et al., 2021). By using bio-stimulants, the damaging effects of many stressors, including As stress, may be reduced (Khan et al., 2021). However, among the several defence metabolites produced naturally by plants, melatonin, as a vital biostimulant, is believed to counteract As stress (Abdollahzade et al., 2021). Plant scientists have currently concentrated on researching the MT-triggered metabolic functions in plants by exogenously supplemented MT (Zhang et al., 2021; Sarioğlu and Kaya, 2022) or increasing internal MT concentrations in genetically-modified plants (Arnao and Hernández-Ruiz, 2021).

Hydrogen sulphide (H_2S) serves an ample purpose as a signalling substance in multiple physiological systems in plants (Liu et al., 2021), such as ROS detoxification (Paul et al. 2020), photosynthesis (Arif et al., 2021) and improvement in the AsA-GSH cycle (Alsahli et al., 2021). The potential of H_2S to minimize stress-induced damage has been recorded in alfalfa under water stress conditions (Antoniou et al., 2020), rapeseed under salinity stress (Cheng et al., 2022) and under As stress in a few plant species, like as rice (Mishra and Singh, 2021), and bean (Siddiqui et al., 2021). To our knowledge, there has been no published report on the contribution of H_2S to arsenic MT-induced toxicity tolerance in pepper plants. In this regard, we hypothesized that combined application of H_2S and MT would be more effective than using them alone in improving As stress tolerance of pepper plants. Our major goal was to investigate the potential role of both H_2S and MT in regulation of essential physio-biochemical processes involved in pepper plant stress resistance.

2. Materials and methods

2.1. Plant cultivation and various treatments

A greenhouse experiment was conducted with pepper (*Capsicum annuum* L. cv. Semerkand). A solution of NaOCl (1%) was used to sterilise the pepper seeds before sowing. Three seedlings were potted in each container (5 L) containing perlite. The greenhouse's environmental conditions, such as night (13 h) and day (11 h) temperatures were kept constant at 10 °C and 20 °C, respectively; these temperatures were suitable for the normal growth of pepper. The relative humidity in the greenhouse was kept between 65% and 70%. All critical nutrients were present in the half-strength Hoagland nutritient solution (NS) (nitrogen: 242 mg L^{-1} as $Ca(NO_3)_2{\cdot}4H_2O$, KNO_3 and NH_4NO_3; phosphorus: 31 mg L^{-1} as KH_2PO_4; potassium: 232 mg L^{-1} as KH_2PO_4 and KNO_3; calcium: 224 mg L^{-1} as $Ca(NO_3)_2{\cdot}4H_2O$; magnesium: 49 mg L^{-1} as $MgSO_4{\cdot}7H_2O$; boron: 0.45 mg L^{-1} as H_3BO_3; copper: 0.02 mg L^{-1} as $CuSO_4{\cdot}5H_2O$; manganese: 0.5 mg L^{-1} as $MnCl_2{\cdot}4H_2O$; molybdenum: 0.0106 mg L^{-1} as $Na_2MoO_4{\cdot}2H_2O$; zinc: 0.48 mg L^{-1} as $ZnSO_4{\cdot}7H_2O$; and iron: 0.5% of $(NH_4)_5$ $[Fe(C_6H_4O_7)_2]$ (Hoagland and Arnon, 1950). A little quantity of a solution of 0.01 M KOH was used to adjust the NS's pH at 5.5. Three replications, each with five pots of each treatment were set-up in a randomly complete block layout.

A week after germination, the plants were foliar applied with melatonin (0.1 mM MT) daily for three days prior to the start of the As stress (AsS) treatments. The solution of MT (Sigma) was prepared in a 0.01% surfactant solution of tween-20. An equal amount of the surfactant solution was sprayed to the control plants as well. Before transplanting into pots, the roots of the pertinent group of plants were maintained in a scavenger of hydrogen sulphide (H_2S), hypotaurine (a 250 mL of 0.1 mM HT) solution for 12 h as done in an earlier study (Kaya, 2021). The seedlings were exposed to control (no As) or arsenic stress (AsV–S) in the form of sodium hydrogen arsenate heptahydrate ($Na_2HAs_4O{\cdot}7H_2O$) through the nutrient solution (NS) for another 14 days. During the stress treatment, sodium hydrosulphide at a concentration of 200 μM (NaHS, a H_2S donor) was supplied through NS. The arrangement of the applied treatments to examine the impact of arsenate (AsV) on pepper plants is shown in Fig. 1A.

To calculate dry weight, three plants per treatment (one from each replicate) were taken out. For calculating dry weights, plants were

picked, air-dried, oven-heated for 10 min at 105 °C, and then kept at 72 °C for an additional 72 h. In order to assess the following parameters, the remaining two plants in each replicate, i.e., six plants in each treatment, were uprooted from the pots.

2.2. Chlorophyll contents and chlorophyll fluorescence

Using the methodology of Strain and Svec (1966), the amount of chlorophyll (Chl) in all harvested samples was measured. A sample of fresh leaf (1.0 g) was triturated using 5 mL of a 90% (v/v) acetone solution and properly filtered. The filtrate was kept in tubes with aluminium sheets tightly wrapped around them to protect them from light. The concentration of Chl *a* and Chl *b* was computed after the sample solutions were read using a spectrophotometer at wavelengths of 663.5 nm and 645 nm, respectively. Three replicates of each treatment were used, and each replication was the average of six readings.

Chlorophyll fluorescence was measured on a leaf that had been acclimated in the dark for 30 min. To measure *Fv/Fm* ratios, a Mini-PAM chlorophyll fluorometer (Walz, Germany) was used. Six readings for each replicate were taken and then averaged for each treatment.

2.3. Calculation of leaf water potential and leaf relative water content (RWC)

To measure RWC, Yamasaki and Dillenburg's (1999) protocol was used. To calculate fresh mass (FM), two leaves from the middle of the plants were removed from each replication and weighed. The turgid mass (TM) was then determined by soaking the leaf samples in distilled water within a closed Petri plate. Finally, to calculate dry mass (DM), the same samples were autoclaved at 80 °C for 48 h. The RWC was computed using the following formula:

$$RWC\ (\%) = (FM - DM)/(TM - DM) \times 100$$

The water potential of leaves was appraised early morning using a fresh enlarged leaf from a plant using the PMS-600 (USA).

2.4. Free proline content in leaves

The colorimetric assay using the ninhydrin method of Bates et al. (1973) was used to analyse the leaf free proline content. A volume of 10 mLof aqueous sulfosalicylic acid (3%) was used to macerate 0.5 g of fresh leaf tissue, and the extracted solution was then thoroughly filtered. An equal volume (2 mL each) of glacial acetic acid (GAA) and ninhydrin made in an acid medium were mixed with two mL of the filtered solution. For 60 min, the samples were thoroughly agitated in a warm water bath (80 °C). The process was then stopped by keeping the samples in an ice bath. After that, the sample solution was mixed with 4 mL of toluene and vortexed for 15–20 s. After pipetting out the chromophore, its absorbance was determined at 520 nm using a spectrophotometer.

2.5. Hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents

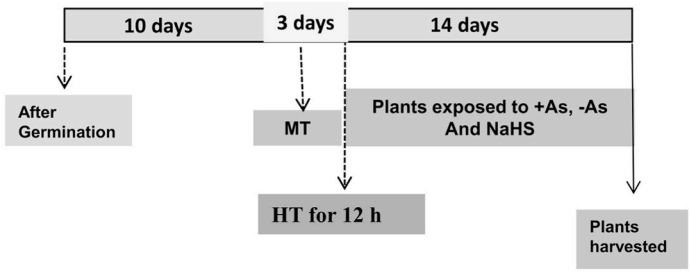
Measurement of leaf H_2O_2 was done following the procedure outlined by Loreto and Velikova (2001). Fresh leaf tissue weighing 500 mg was homogenised in 3 mL of trichloroacetic acid at 1%. (TCA). The homogenised material underwent a 10-min centrifugation at 10,000 *g* and 48 °C. A 0.75 mLof the aliquot was then mixed with 0.75 mL of 10 mM K buffer and 1 mL of KI (1.5 mL). At 390 nm, the treated samples' optical densities (OD) were measured.

The procedure outlined by Weisany et al. (2012) was followed to calculate the leaf MDA. Five mL of trichloroacetic acid (TCA; 0.1% w/v) were used to extract 0.2 g of fresh leaf. The mixture was then centrifuged at 12,000 *g* for 5 min. Thiobarbutaric acid (TBA; 4 mL, 0.5%) was used to treat the sample solution with TCA (20%). In a water bath, the mixture was heated at 90 °C for 30 min. Before determining their OD at 532 nm, the treated samples were properly chilled.

2.6. Electrolyte leakage (EL)

EL was evaluated using the previously improved approach by Dionisio-Sese and Tobita (1998). To get rid of any contamination on the surface, leaf tissues were rinsed with deionized water. To test the initial

A



B

Fig. 1. An outline of the treatments of different chemicals applied to examine the effect of Arsenic (As) on pepper plants. The concentration of each chemical used was 50 μM As(V)–S) supplemented without or with 0.10 mM melatonin (MT) and 200 μM sodium hydrosulphide (NaHS, an H_2S donor)) singly or jointly with a scavenger of H_2S, hypotaurine (0.1 mM HT). (B) Effects of MT and NaHS alone or together, or combined with HT on the growth of pepper plants. Photographs were taken at harvest stage **1: C, 2: As–S, 3: As + MT, 4: As + MT + NaHS, 5:As + MT + HT, 6: As + MT + NaHS + HT.**

electrolyte conductivity (EC1), leaf discs from the fresh leaf tissues were placed in vials with 10 mLof deionized water and shaken at room temperature for 24 h. To evaluate the final electrolyte conductivity (EC2), the samples with leaf tissue were then autoclave-heated for 20 min at 120 °C. EL was determined via the formula shown below:

$$EL\ (\%) = (EC1/EC2) \times 100$$

2.7. Quantification of subcellular As distribution

The method used by Sheng et al. (2016) was used to measure the subcellular fractionation of As in leaf and root tissues. In a 10 mL of the extraction solution (0.25 mM sucrose, 1.0 mM DL-dithioerythritol, 50 mM Tris-HCl and 5 mM ascorbic acid), fresh leaf tissue (0.5 g) was extracted quickly. A nylon filter with a mesh size of 100 mm was used to filter the extract, and the filtrate was designated as "cell wall fraction" (CWF) and centrifuged for 45 min at 20,000 g. The aliquot solution was labelled as the "soluble fraction" (SF) and the resulting pellet as the "organelle fraction" (OF). This work was done at 4 °C. In a 100 mL Erlenmeyer flask with de-ionized water, CWF and OF were both transferred, dried, and digested with HNO_3 (5 mL) (Su et al., 2014). The As concentrations in all treated samples were then evaluated using an ICP-OES (PerkinElmer Optima 5300 DV).

2.8. Quantification of phytochelatins

By subtracting the glutathione (GSH) content from the total non-protein thiol (NPT) content, phytochelatin (PC) content was calculated. A 3% solution of sulfosalicylic acid was employed to macerate the tissue of fresh leaves. A 5 mM EDTA and 0.6 mM DTNB [5,5 o-ithiobis (2-nitrobenzoic acid)] were present in the Ellman's reaction mixture. Following Ellman (1959), the NPT's measurement was made at 412 nm.

2.9. Analysis of ascorbate (AsA) and glutathione (GSH)

A fresh leaf sample (500 mg) was used to measure AsA and GSH levels. A 3 mL of cold solution containing 1 M EDTA and 5% metaphosphoric acid was used to extract each sample. After correctly centrifuging the mixture at 11,500 *g*, AsA and GSH measurements were done by pipetting off the supernatant. The procedure of Huang et al. (2005) was followed to quantify the AsA and dehydroascorbate (DHA) contents. A total of 0.6 mL of 0.5 M K-phosphate buffer of pH 7.0 and 100 mM K-phosphate buffer of pH 7.0 containing 0.5 units of ascorbate oxidase were used to neutralize 0.4 mL of the aliquot. The reduced AsA's absorbance readings were taken at 265 nm. The sample extract was processed with 30 mM dithiothreitol (DTT), and the absorbance for the total AsA was measured at 265 nm. To calculate the concentration of DHA, the reduced-AsA concentration was deducted from the total AsA concentration.

The levels of glutathione disulfide (GSSG) and oxidised GSH were determined using the procedure outlined by Yu et al. (2003). With the aid of 0.6 mL of 500 mM K-phosphate buffer of pH 7.0, a 0.4 mLaliquot was neutralised. The variations in the absorbance rate of NTB (2-nitro-5-thiobenzoic acid) at 412 nm caused by the decrease of DTNB (5,5′-dithio-bis 2-nitrobenzoic acid) were used to compute GSH. By eliminating GSH with the use of a derivatizing agent, 2-vinylpyridine, GSSG was determined.

2.10. Hydrogen sulphide (H_2S) measurement

To assay leaf H_2S, the procedure developed by Nashef et al. (1977) was undertaken. A solution of potassium phosphate buffer (0.1 M) was used to macerate the leaf material. The resulting extract was centrifuged at 4 °C for 15 min at 15,000 *g*. For the measurement of H_2S, a 2-mL assay mixture containing an aliquot of 188 μL of the extraction buffer, 100 μL of the extract, and 20 μL of 20 mM 5,5′-dithiobis (2-nitrobenzoic acid) was incubated for 2 min at 25 °C. At 412 nm, absorbances of all treated samples were measured.

2.11. Plant crude extracts

A 500 mg leaf sample was mixed in 1.0 mL of 50 mM at K–P buffer of pH 7.0 that contains 100 mM KCl, 1.0 mM AsA, 5 mM β-mercaptoethanol and 10% (v/v) glycerol in a mortar with a pestle for 10 min. The extract was centrifuged at 10,000 *g* for 10 min at 4 °C. The supernatant was also employed to assess protein content (Bradford, 1976) and the enzyme activities.

2.12. Assays of the glyoxalase system and the ascorbate-glutathione cycle's enzyme activities

The ascorbate peroxidase (APX) activity was evaluated using the method of Nakano and Asada (1981). A cocktail solution made up of 50 mM potassium-phosphate buffer of pH 7.0, 0.1 mM EDTA, and 0.5 mM AsA was mixed with the plant extract (0.7 mL). The reaction was finally started by adding 0.1 mM H_2O_2, and the sample mixture's optical density was measured at 290 nm for 1 min.

In an assay mixture consisting of Tris-HCl buffer (50 mM at pH 7.5), AsA (2.5 mM), NADPH (0.2 mM), ascorbate oxidase (0.5 units) and the plant extract, monodehydroascorbate reductase (MDHAR) was measured in accordance with the method illustrated by Hossain et al. (2010). The mixed solution's optical density was appraised at 340 nm for 1 min after the reaction was started with H_2O_2.

The method refined by Nakano and Asada (1981) was used to measure the activity of dehydroascorbate reductase (DHAR). A cocktail solution containing 0.1 mM DHA, 50 mM K–P buffer, and 2.5 mM GSH was added to the enzyme solution. The sample solutions were then read for absorbance at 265 nm.

Based on method of Hasanuzzaman et al. (2011), glutathione reductase (GR; EC) activity was assessed. The test solution for the enzyme was mixed with 0.1 M K–P buffer, pH 7.0, consisting of 1 mM GSSG, 1 mM EDTA, and 0.2 mM NADPH. Afterward, the change in absorbance at 340 nm was noted.

The technique described by Hasanuzzaman et al. (2014) was used to measure glyoxalase I (Gly I) activity. The enzyme test solution was supplemented with a cocktail solution consisting of 100 mM K–P buffer at pH 7.0, 1.7 mM GSH, 15 mM magnesium sulphate and 3.5 mM MG. At 240 nm, absorbance measurements were taken. The method outlined by Hasanuzzaman et al. (2014) was employed to assess the activity of glyoxalase II (Gly II). The enzyme test solution was mixed with a cocktail solution comprising 1 mM S-D-lactoylglutathione (SLG), 0.2 mM DTNB, and 100 mM Tris-HCl buffer at pH 7.2. Following that, the absorbance readings at 240 nm were noted, and the Gly-II activity was calculated.

2.13. Assays for other antioxidant enzymes' activities

In a mortar and pestle, 0.5 g of fresh leaf material were extracted with 50 mM Na–P (pH 7.8) buffer that included 1% soluble polyvinylpyrrolidone. After that, the homogenate was properly centrifuged at 20,000 *g*. The superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) activitiesy were assessed using the aliquot. The SOD's capacity to prevent nitroblue tetrazolium (NBT) from undergoing photochemical inhibition served as a measure of its activity (Van Rossum et al., 1997). The amount of enzyme needed to lower the amount of cytochrome *c* by 50% was the SOD activity, which was given on a per-unit basis. Following the removal of H_2O_2, the CAT activity was measured as a suppression in optical density at 240 nm for 60 s (Chance and Maehly, 1955). The elevation in optical density at 470 nm caused by the guaiacol oxidation by H_2O_2 was used to measure the POD activity (Chance and Maehly, 1955).

2.14. Methylglyoxal (MG) levels

After extracting 500 mg of leaf tissue in 5% perchloric acid, the homogenised solution underwent centrifugation at 11,000 *g* at 4 °C for 10 min. The supernatant was decolored by the addition of charcoal, and at 25 °C, it was neutralised by the addition of a saturated potassium carbonate solution. A solution of *N*-acetyl-L-cysteine and sodium dihydrogen phosphate (at the ratio of 24:25:1) was used to dilute the reaction mixture to a final volume of 1 mL. At a wavelength of 288 nm, after 10 min, the end-product *N*-acetyl-S-(1-hydroxy-2-oxo-propyl) cysteine was detected (Wild et al., 2012).

2.15. Statistical analysis

Data for each parameter was subjected to a two-way of analysis of variance for calculating variance in each data set using the SAS version 9.1 (SAS Institute Inc, NC, USA). The Duncan's Multiple Range test was used to compare the mean values, and the alphabets on bars show if the mean values were significantly different at the 0.05% level of probability. Each tested parameter was run three times.

3. Results

3.1. H_2S produced by MT promotes phenotypic appearance of the pepper plants under As stress

The pepper plants exposed to As and other compounds showed no change in colour in the shoots. Under As stress, the pepper plants' plant height and leaf size clearly reduced, as shown in Fig. 1B. Moreover, after a few days of As treatment alone, the pepper seedlings began to wilt. The As-induced reduction in plant height and leaf size was reversed in the plants treated with MT, and MT + NaHS. However, HT abolished the

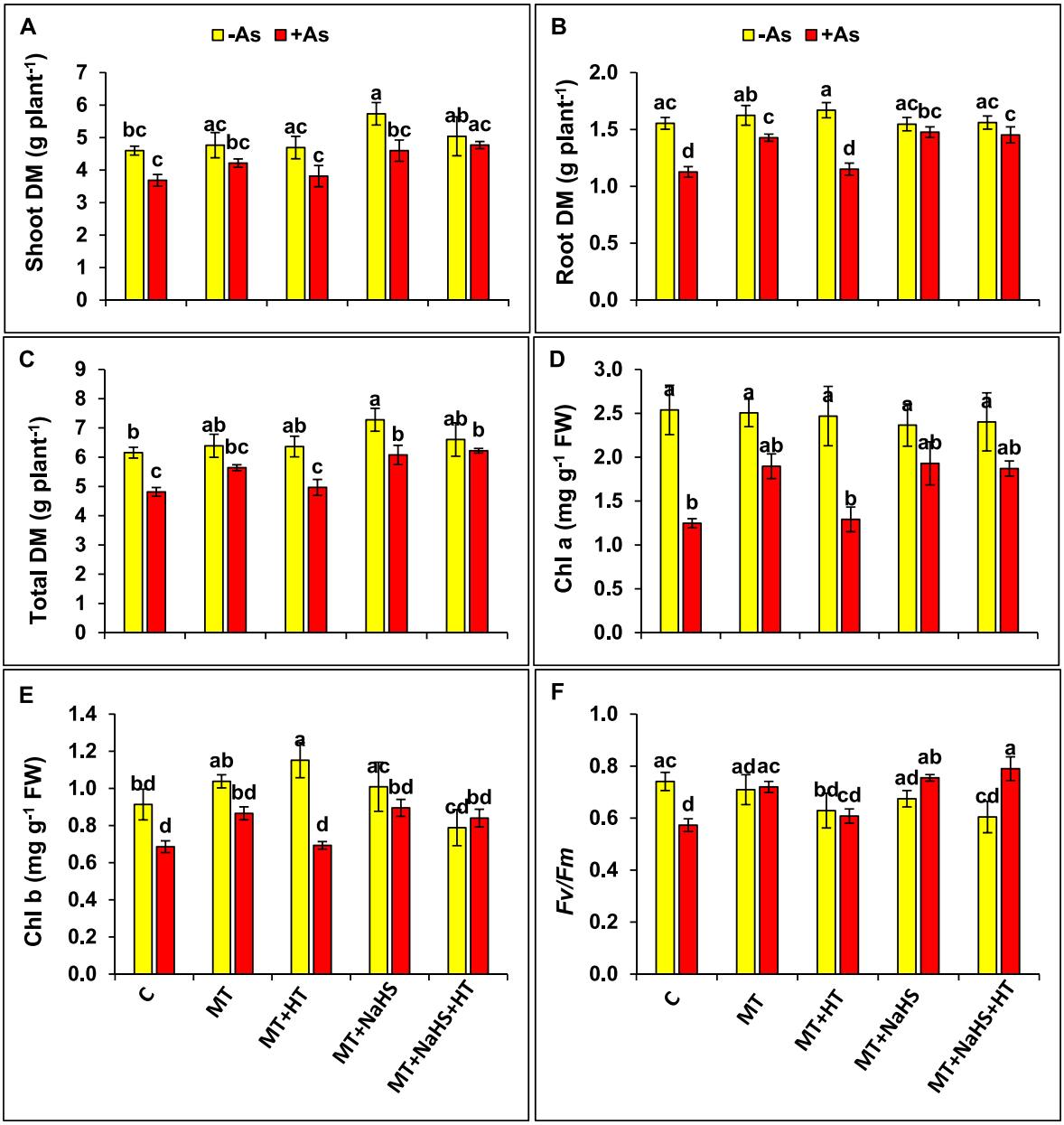


Fig. 2. Shoot (A), root (B) and total (C) dry matter (DM), chlorophyll *a* (D), chlorophyll *b* (E) on fresh weight (FW) basis and chlorophyll fluorescence parameters [*Fv/Fm* (F)] in arsenic (As)-stressed pepper plants sprayed with 0.10 mM melatonin (MT) and 0.20 mM sodium hydrosulphide (NaHS, an H_2S donor)) singly or jointly with a scavenger of H_2S, hypotaurine (0.1 mM HT). Mean ± S.E; Different alphabets on bars reflect that the mean values differ significantly at $P \leq 0.05$.

beneficial impact of MT on the visible signs of AS-stressed plants. But the addition of NaHS together with HT + MT somewhat reversed the negative impact of HT on worsening visual symptoms.

3.2. H_2S produced by MT promotes pepper plant growth

To estimate plant growth, data for dry weight of shoot, root, and total plant were employed (Fig. 2A–C). When compared to the control plants, arsenic stress (AsS; 50 M) substantially ($P \leq 0.05$) reduced the shoot, root, and total plant dry mass by 23.7%, 28.9%, and 24.4%, respectively. However, externally applied 0.10 mM melatonin (MT) significantly increased plant growth-related parameters by 16.1%, 17.1%, and 16.3%, respectively, in comparison with those in the As stressed-plants alone (Fig. 2A–C). Further elevations of these variables by 26.5%, 29.2%, and 27.6%, respectively, with respect to those in the As stressed-plants alone were induced by MT and sodium hydrogen sulphide (NaHS), a source of hydrogen sulphide (H2S). The application of MT jointly with hypotaurine (0.1 mM HT, a scavenger of H2S) treatment reduced the beneficial effects of MT on plant traits in the AsS-plants. However, the application of NaHS along with MT restored the H2S synthesis and restored the favourable impact of MT on these parameters. These results demonstrate that for MT to be effective on plant growth, H2S production is necessary. As a result, both H2S and MT contributed to the improvement of arsenic tolerance in pepper plants. The fact that no other treatments significantly changed these traits in the controls shows that MT or HT was ineffective in controlling plant growth without stress. . As a result, MT and H2S together improved pepper plant growth when exposed to As–S.

3.3. H_2S produced by MT boosts photosynthesis-related parameters in pepper plants

Unlike non-stressed plants, arsenic toxicity considerably ($P \leq 0.05$) reduced chlorophyll *a and b* concentrations as well as photosystem II efficiency (*Fv*/*Fm*) in the pepper plants by 51.7%, 25.9%, and 25.0%, respectively. In contrast, MT and MT + NaHS enhanced chlorophyll *a* by 52.2% and 55.0%, chlorophyll *b* by 28.2% and 30.92%, and *Fv*/*Fm* by 21.72% and 26.72%, respectively, compared with those in plants that have only been exposed to AsS (Fig. 2D–F). By likely reducing H2S production, the treatment of HT together with MT negated the positive effects of MT on these attributes in the As–S-plants. The unfavourable effects of HT combined with MT under As–S were reversed by the NaHS treatment (MT + HT + NaHS). This suggests that the production of H2S is necessary for MT-induced enhanced photosynthesis under As–S. As a result, H2S and MT together increased the pepper plants' ability to tolerate As–S.

These attributes of the untreated control plants remined unaffected by the treatments; this implies that neither HT nor MT had a harmful or beneficial effect on the attributes related to photosynthesis in the non-stressed plants.

3.4. H_2S produced by MT improves leaf water relations and proline content in pepper plants

Fig. 3 shows the results for leaf RWC, water potential (ΨI), and proline. Arsenic stress substantially raised the proline content by 266.8% compared to that of the control by reducing leaf RWC and leaf ΨI by 24.7% and 162.9%, respectively (Fig. 3A–C). In contrast to those in the As stressed-plants, pre-treatment with MT and MT + NaHS raised the leaf RWC by 25.1% and 28.9%, leaf ΨI by 16.9% and 22.5%, and proline content by 41.8% and 82.7%, respectively. According to the results, MT and NaHS together (MT + NaHS) were more beneficial in enhancing water relation metrics and proline content than by the MT pre-treatment alone. The ameliorating effects of MT alone on these attributes were inverted by the addition of HT, while those of MT + NaHS were not, indicating that H_2S was perhaps involved in the enhancement of MT-induced improvements in water related attributes. These attributes remained unchanged after several treatments on the non-stressed plants.

3.5. Pretreatment of MT enhances H_2S synthesis and declines oxidative stress

Changes in the H2S synthesis were also assessed to get an understanding of the potential role that H2S may have played in the MT-induced improvement in the pepper plants' ability to tolerate As stress. Compared to the controls, the As–S-plants showed a small reduction in H_2S synthesis (16.3%) (Fig. 3D). However, H2S synthesis increased under As–S with the addition of MT and MT + NaHS. The HT's pre-treatment turned the H2S concentration down. The negative effects of HT on H2S concentration were abolished by the supply of NaHS and MT.

H_2O_2 (3.6-fold), MDA (3.5-fold), and electrolyte leakage (EL, 2.4-fold) contents, which are oxidative stress-related attributes, were considerably ($P \leq 0.05$) elevated in the pepper plants under As–S (Fig. 3E–G). Compared to the As–S-plants alone, MT treatment decreased the oxidative stress-related parameters by 41%, 31%, and 33%, respectively. Additionally, the As–S-plants showed higher decreases in these attributes after receiving MT and NaHS supplements. The favourable effects of MT applied alone were reversed by the HT, but not those of MT when applied jointly with NaHS on the oxidative damage; this could be that HT prevented the H_2S production.

3.6. H_2S produced by MT regulates subcellular As content in pepper plants

In the root and leaf tissues of the As–S-plants, there was a noticeable ($P \leq 0.05$) increase in sub-cellular As accumulation. The absence of arsenic in the root zone was primarily responsible for the absence of arsenic from the leaf and root of the unstressed plants. As–S caused a significant As accumulation in the root cell walls (57%) and soluble fraction (in vacuoles; 31%) as well as cell organelles (12%) (Fig. 4A). However, the MT treatment increased the amount of soluble As in the root vacuoles. A further increase in the soluble fraction of As was induced by the addition of NaHS and MT (MT + NaHS). These results unambiguously show that MT reduced As accumulation at the root surfaces and increased As's solubility in the vacuoles, which might have lessened the As harmful effects on cells. Arsenic was deposited mostly in the cell wall (51%), followed by that in soluble fraction of vacuoles (35%) and cell organelles (14%) in the leaves of the As–S plants. (Fig. 4B). The subcellular leaf As content of the As–S-pepper plants increased in the cell wall and vacuoles, but reduced in the cell organelles with the addition of MT and MT + NaHS. These results showed that compared to that in the As stressed plants, the As content in the leaves increased due to increased As content in the cell walls and vacoulles due to MT and MT + NaHS treatment, and these exogenous treatments enhanced the deposition and fixing of the majority of As in the leaf cell wall and vacuoles, hence promoting the reduction of As being transferred to the other leaf cell organelles. On the other hand, by lowering the generation of H2S, pretreatment of HT eliminated this regulating role of MT on the subcellular distribution of As. This means that endogenous H_2S is crucial for limiting As absorption and for causing the sequestration of absorbed As into vacuoles, so, H2S may be a downstream molecular indicator of MT-induced tolerance of pepper plants to As–S.

3.7. H_2S produced by MT enhances accumulation of phytochelatins, GSH and AsA

Significant increases in phytochelatin synthesis (PC, 6.5-fold) were observed in plants grown in As–S. (Fig. 5A). Additionally, they had higher levels of GSH (56%) and GSSG (50%), but a lower GSH/GSSG

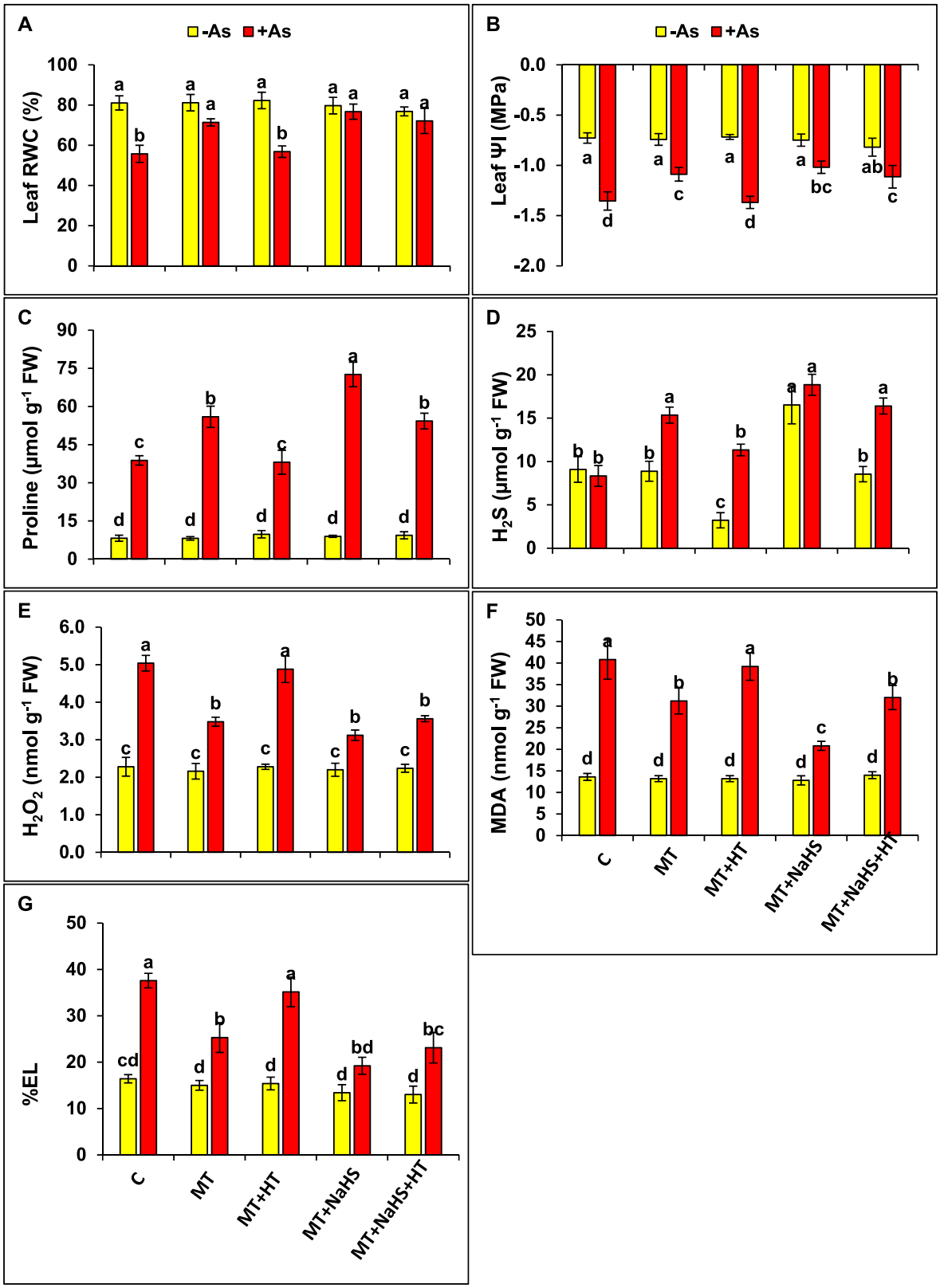


Fig. 3. Leaf relative water content [RWC; (A)], leaf water potential [Leaf Ψl; (B)], proline (C), hydrogen sulphide [H_2S (D)], hydrogen peroxide content [H_2O_2; (E)] and malondialdehyde [MDA; (F)] on fresh weight basis and electrolyte leakage [EL (G)] in arsenic (As)-stressed pepper plants sprayed with 0.10 mM melatonin (MT) and 0.20 mM sodium hydrosulphide (NaHS, an H_2S donor)) singly or jointly with a scavenger of H_2S, hypotaurine (0.1 mM HT). Mean ± S.E; Different alphabets on bars reflect that the mean values differ significantly at $P \leq 0.05$.

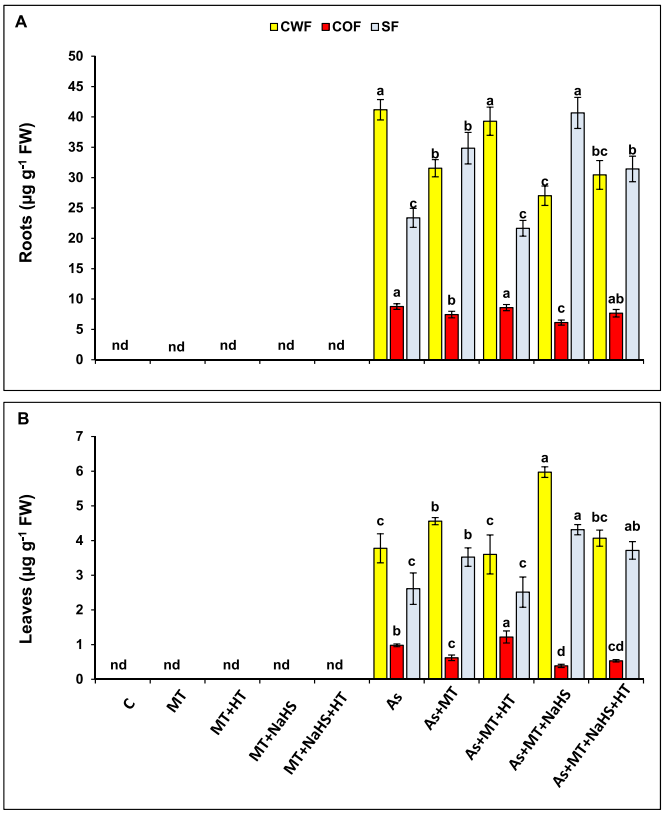


Fig. 4. The cell wall (CW), cell organelle (CO) and soluble fractions (SF) of arsenic in root (A) and leaf (B) in arsenic (As)-stressed pepper plants sprayed with 0.10 mM melatonin (MT) and 0.20 mM sodium hydrosulphide (NaHS, an H_2S donor)) singly or jointly with a scavenger of H_2S, hypotaurine (0.1 mM HT). Mean ± S.E; Different alphabets on bars reflect that the mean values differ significantly at $P \leq 0.05$.

ratio (Fig. 5B–D) than those recorded in the plants grown normally. With the exception of a decrease in GSSG, all these parameters increased as a result of the supply of MT and As–S. These features responded more well to the combination of MT and NaHS treatment. This implies that MT alone or in combination with NaHS may lead to As detoxification by increasing the synthesis of PC and GSH.

AsA levels significantly decreased (by 23%), but DHA levels increased (by 34%) in the As–S-plants compared to those in the plants receiving normal chemical treatments (Fig. 5E and F). As a result, compared to the controls, the plants exposed to As–S had a 42% lower ratio of AsA/DHA (Fig. 5G). The As–S plants treated with MT or MT + NaHS had higher AsA concentrations and AsA/DHA ratio than those of the As–S plants treated with no additional chemical, but their DHA content was significantly lower.

On the other hand, by reducing H_2S generation, the HT treatment reversed the up-regulatory impact of MT on all of the aforementioned parameters, but in the As–S plants, the lowering effect of HT on H_2S synthesis was abolished by the addition of MT together with NaHS. These results suggest that enhanced H_2S generation in the As–S pepper plants is the most likely cause of beneficial effects of MT on As–S plants.

3.8. H_2S produced by MT upregulates antioxidant enzyme activities

In pepper plants exposed to AsS, superoxide dismutase (SOD) activity was considerably boosted by 2.8-fold, although catalase (CAT) and peroxidase (POD) activity was significantly ($P \leq 0.05$) decreased over that in the non-treated plants by 3.2- and 2.9-fold, respectively (Fig. 6A–C).

MT and MT + NaHS treatments reduced the SOD activity, while increased that of CAT and POD. These enzymes' activities were reversed by the administration of HT, which demonstrates that MT caused H_2S to modulate antioxidant activities in the presence of As–S. By restoring H_2S production, the addition of NaHS and MT (MT + NaHS) totally reversed the detrimental effects of HT on these enzymes' activity.

3.9. H_2S produced by MT upregulates the AsA-GSH cycle enzymes

The GR, APX, MDHAR, and DHAR activities of the AsA-GSH cycle related enzymes were assessed so as to determine the role of H2S in the MT-induced modification of As–S tolerance of pepper plants. Compared to those in the control plants, arsenic stress significantly ($P \leq 0.05$) increased the APX and GR activities by 2.3- and 1.8-fold, but reduced those of MDHAR and DHAR by 2.2- and 1.6, respectively (Fig. 7A–D). All of these enzymes' activities were increased in the As–S plants by the addition of MT or MT + NaHS.

The beneficial effect of MT on these enzymes' activities was eliminated by supplementation of HT to the As–S plants, perhaps by decreasing H_2S production. This demonstrates that MT needs H_2S to stimulate the AsA-GSH cycle. The supplementation of NaHS plus MT (MT + NaHS) totally reversed the negative effects of HT on the activities of these enzymes by restoring H_2S synthesis.

H_2S produced by MT decreases methylglyoxal (MG) levels and increases the activity of the glyoxalase system enzymes.

In response to AsS, the content of MG was noticeably increased by 2.4-fold compared to that in the control plants (Fig. 7A). In contrast to the untreated plants, the As-stressed plants had considerably lower the enzymes' activities connected to the glyoxalase system (Fig. 7B and C).

In the As-treated plants, the use of MT and MT + NaHS led to a considerable reduction in MG (by 20% and 29%, respectively). Additionally, when compared to the plants solely supplied with As, the MT and MT + NaHS treatments increased Gly I by 37% and 73%, and Gly II activities by 27% and 40%, respectively. However, the positive effect of MT was abolished by the HT supply, because of reduced synthesis/accumulation of endogenous H_2S. The negative effects of HT on these attributes were totally inverted by the combined application of NaHS and MT (MT + NaHS), demonstrating that, under AsS regimes, endogenous H_2S was necessary for MT-induced activation of the glyoxalase system and reduction of MG content.

4. Discussion

Arsenic stress is one of the principal abiotic factors impeding plant growth and damaging human health (Shabbir et al., 2021). Previous studies have demonstrated that external application of certain bioactive molecules may assist to lessen the detrimental effects of a variety of abiotic stresses (Arnao and Hernández-Ruiz, 2014; Drobek et al., 2019; González-Morales et al., 2021). Melatonin (MT) has recently attracted a considerable interest in plant biology (Murch and Erland, 2021). Due to its clear physiological functions, MT is referred to as a potential biostimulant (Moustafa-Farag et al., 2020; Hoque et al., 2021). Although MT has been shown to work well in reducing the adverse effects of As–S in some plants, including rice (Nazarian and Ghanati, 2020; Bao et al., 2021), and rapeseed (Farooq et al., 2022), no research has been done on the potential contribution of H_2S production to stress tolerance in plants caused by MT. Thus, the main goal of this investigation was to ascertain whether or not H_2S accumulation or synthesis plays a role in pepper plants' ability to tolerate As stress when MT was applied.

4.1. The endogenous H_2S is required for MT-induced enhanced photosynthesis-related attributes in As-stressed plants

Parallel to what has already been demonstrated in *Pteris cretica* (Zemanová et al., 2020) and rice plants (Ghorbani et al., 2021; Faizan et al., 2021), in the current research As–S inhibited photosynthesis-related characteristics, chlorophyll content, and

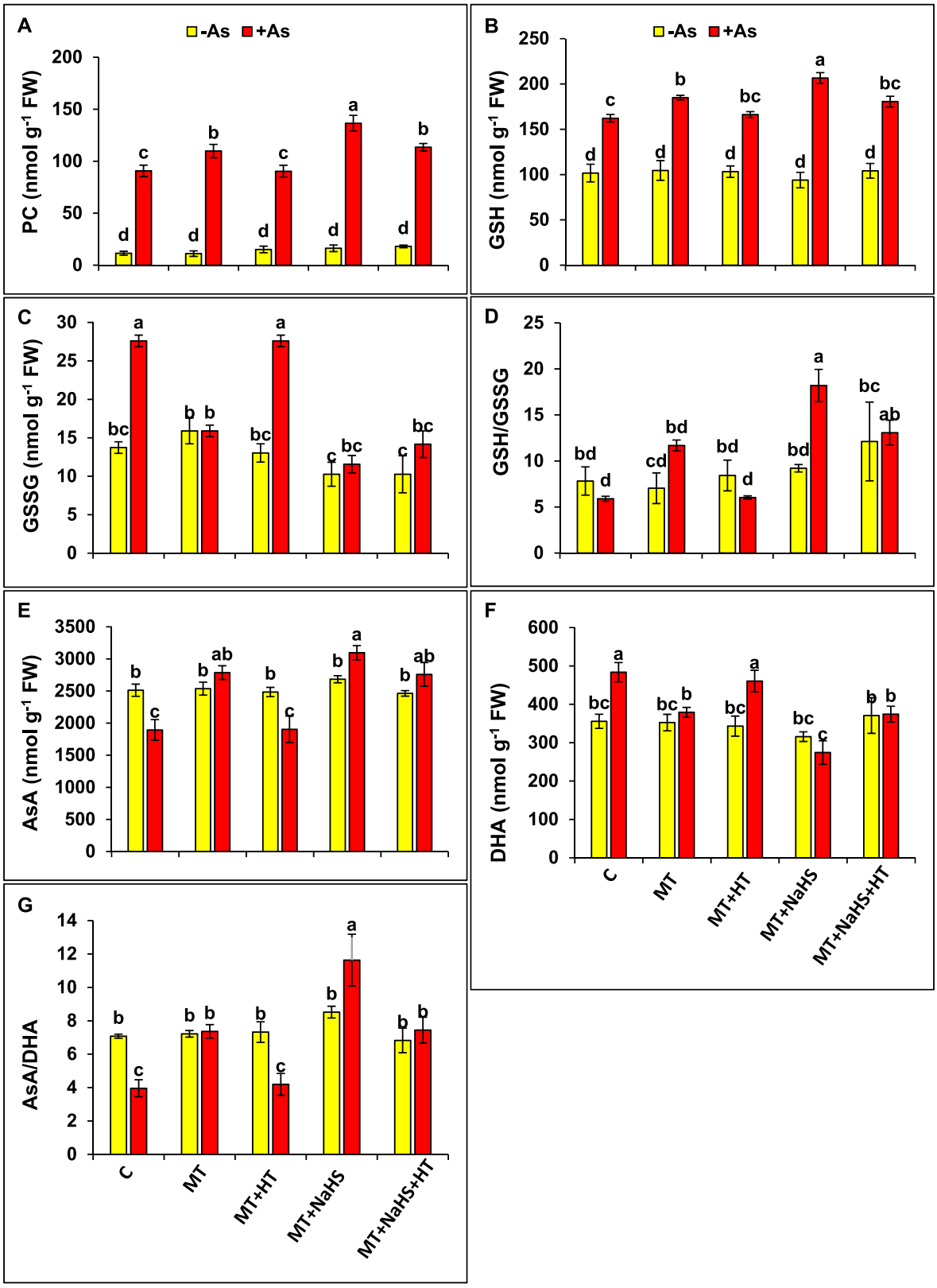


Fig. 5. Phytochelatins [PC (A)], reduced glutathione [GSH (B)], oxidised glutathione [GSSG (C)] on fresh weight (FW) basis, and GSH/GSSG (D), Ascorbate [AsA (E)], and dehydroascorbate [DHA (F)] on fresh weight (FW) basis, and AsA/DHA ratio (G) in arsenic (As)-stressed pepper plants sprayed with 0.10 mM melatonin (MT) and 0.20 mM sodium hydrosulphide (NaHS, an H_2S donor)) singly or jointly with a scavenger of H_2S, hypotaurine (0.1 mM HT). Mean ± S.E; Different alphabets on bars reflect that the mean values differ significantly at $P \leq 0.05$.

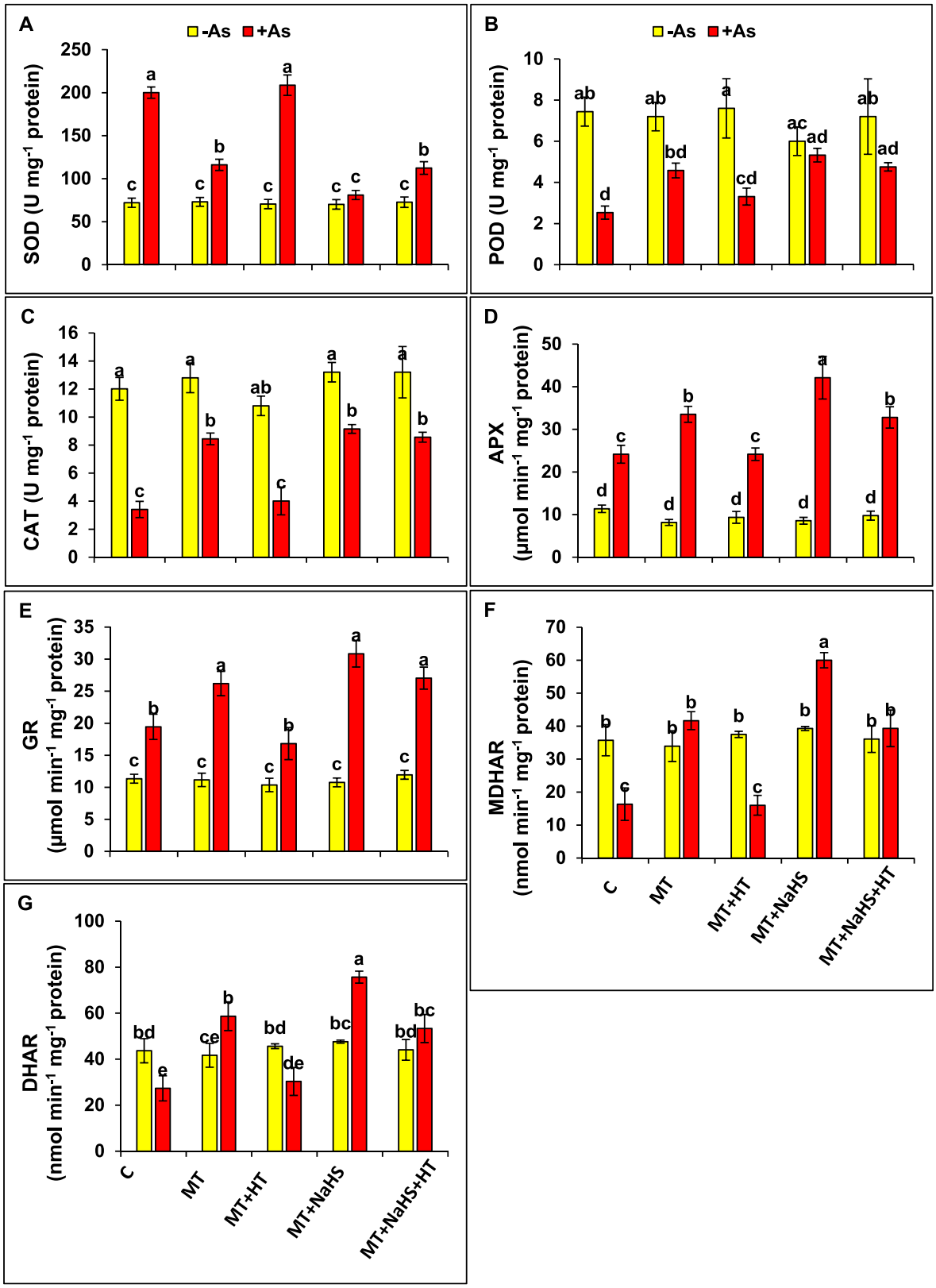


Fig. 6. Superoxide dismutase [SOD (A)], peroxidase [POD (B)], catalase [CAT (C)] and ascorbate peroxidase [APX (D)] activies, glutathione reductase [GR (E)], dehydroascorbate reductase [DHAR (F)] and monodehydroascorbate reductase [MDHAR (G)] in arsenic (As)-stressed pepper plants sprayed with 0.10 mM melatonin (MT) and 0.20 mM sodium hydrosulphide (NaHS, an H_2S donor)) singly or jointly with a scavenger of H_2S, hypotaurine (0.1 mM HT). Mean ± S.E; Different alphabets on bars reflect that the mean values differ significantly at $P \leq 0.05$.

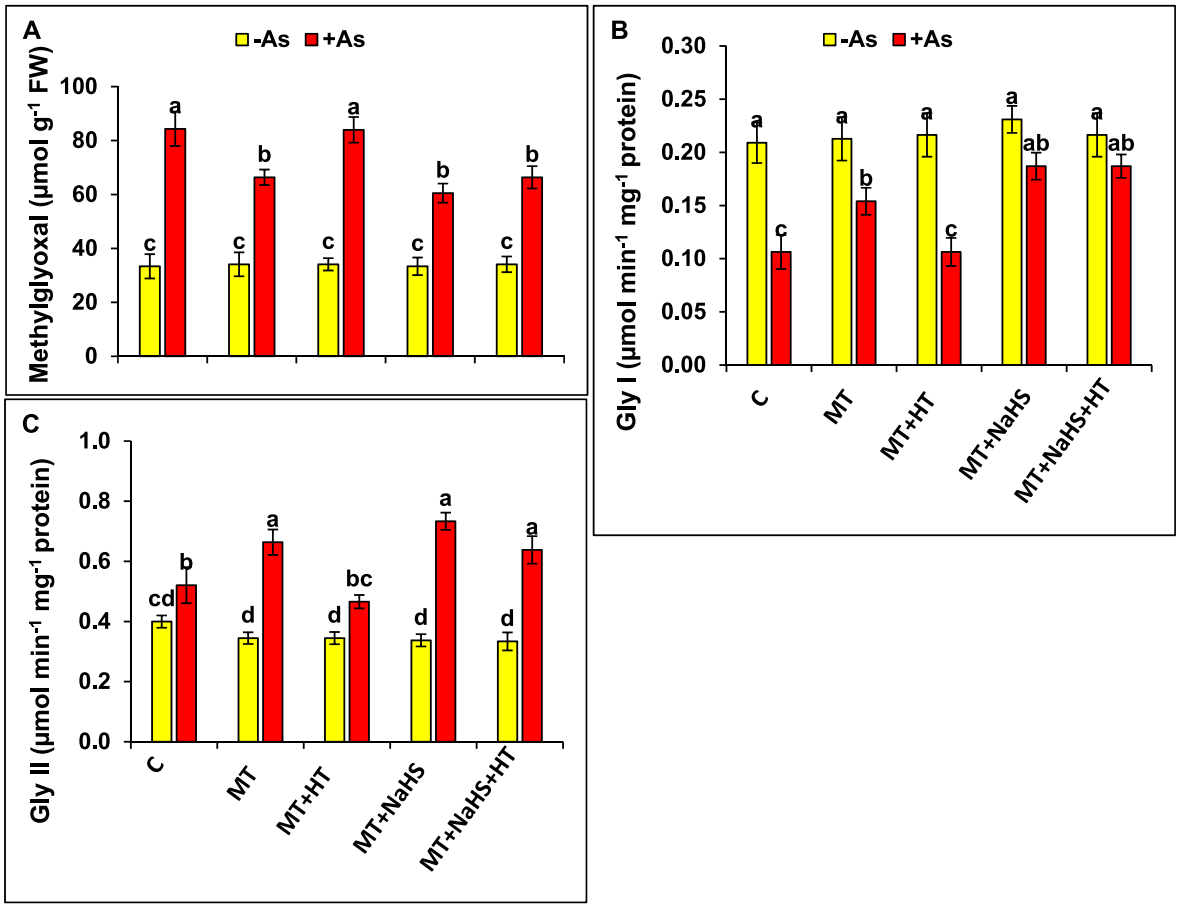


Fig. 7. Methylglyoxal [MG (D)] content, glyoxalase I [Gly I (E)] and glyoxalase II [Gly II (F)] in arsenic (As)-stressed pepper plants sprayed with 0.10 mM melatonin (MT) and 0.20 mM sodium hydrosulphide (NaHS, an H_2S donor)) singly or jointly with a scavenger of H_2S, hypotaurine (0.1 mM HT). Mean ± S.E; Different alphabets on bars reflect that the mean values differ significantly at $P \leq 0.05$.

maximal photochemical efficiency (*Fv/Fm*) in the pepper plants. However, MT pre-treatment improved all attributes associated with photosynthesis under As–S, as previously recorded in pepper (Kaya et al., 2022) and rapeseed (Farooq et al., 2022) plants. Improved photosynthesis-related characteristics and decreased H_2O_2 content as a result of the application of MT imply that MT helped mitigate the negative impact of AsS on traits associated with photosynthesis, likely by lowering H_2O_2 production, as previously shown in rapeseed plants (Farooq et al., 2022). As was already established in our experiment, the pepper plants' tolerance to As stress was increased via MT-mediated synthesis of intrinsic H_2S. By lowering H_2S build-up, HT supply, however, counteracted the positive effects of MT on all parameters related to photosynthesis. The pre-treatment of MT + NaHS offers an additional information demonstrating that this co-treatment resulted in further rises in *Fv/Fm* and chlorophyll content; these results are comparable to those stated earlier (Alsahli et al., 2021) wherein exogenously supplied NaHS enhanced the amount of chlorophyll in pea plants.

4.2. *In As-stressed plants, MT promotes plant growth, water relations, and proline, all of which depend on endogenous H_2S supplementation*

Disruption in plant-water relationships is one of AsS's main negative impacts on plants (Bali and Sidhu, 2021). It has also been reported elsewhere that one mechanism for reducing As absorption and transmitting it to plant shoots is the lowered water status caused by As in wheat plants (Maghsoudi et al., 2020). Several plant species experience root and leaf desiccation as a result of decreased water uptake brought by heavy metals or metalloids (Noor et al., 2022; Ghorai et al., 2022). However, plants have developed an adaptive mechanism to boost water absorption from the growing medium through the production and/or buildup of osmolytes like proline (Dien et al., 2019; El Moukhtari et al., 2020), similar to what we observed in our research, wherein AsS raised the proline concentration. In order to preserve the plant's water status, proline content possibly rose under AsS conditions, similar to what has been found in tomato wherein AsS reduced leaf RWC and increased proline content (Kaya and Ashraf, 2022).

The reports on rapeseed (Farooq et al., 2022) and soybean (Bhat et al., 2022) clearly show that MT treatment was helpful in promoting plant growth under As treatment. In pepper plants exposed to As–S in the current investigation, the pre-treatment with MT or MT + NaHS increased leaf RWC, proline content and Ψl, suggesting that MT might increase water content of plants by improving proline synthesis, as it was previously reported that MT increased the proline content of plants grown under metal stress (Zhang et al., 2020). One of the causes of the improved plant growth after pre-treating with MT under As stress may have been due to the reason that MT decreased the deposition of arsenic in the pepper leaves, as has been previously demonstrated in tea plants (Li et al., 2021).

In order to find out if H_2S contributes to increased tolerance of the pepper plants to AsS due to MT or MT + NaHS the As-treated plants were additionally supplied with HT, a H_2S scavenger, to stop H_2S production by the supplementation of MT. The increased H_2S level caused by MT promoted plant growth under AsS, while in the case of H_2S scavenger, MT exhibited no positive influence on plant growth. As a result, it was proposed that the increased plant growth caused by MT under AsS relied on H_2S synthesis. H_2S may therefore be a downstream signal molecule of

MT under AsS. The treatment of MT + NaHS offers a compelling evidence that it enhanced the concentration of endogenous H_2S to the level that it had been effective in improving MT-induced As stress tolerance. Previous research has shown that exogenously administered NaHS increases As stress tolerance (Siddiqui et al., 2021; Mishra and Singh, 2021). The role of H_2S in the tolerance to stress brought about by MT, however, is not reported in the literature. So, our work offers the first report of the interaction of MT with H_2S in augmenting pepper plants' tolerance to As stress.

4.3. Subcellular root and leaf As distribution in the cell of plants under As–S

As in our investigation in pepper plants, Singh et al. (2015) previously observed that As–S-pea plants produced less H_2S. H_2S production in the As–S-pepper plants improved due to the addition of MT, showing that H_2S functions as a downstream biomolecule signal in MT-treated plants. This may be the cause of the MT-induced reduction in oxidative stress, presumably through activation of the AsA-GSH cycle, which would increase plant tolerance to As–S. It has already been demonstrated that H_2S at safe levels helps plants better endure biotic and abiotic stressors (Shi et al., 2015). MT-generated H_2S synthesis under As–S has not been earlier reported, but under other stresses including heat and saline stress, MT application showed enhanced generation of H_2S, which resulted in improved stress tolerance of wheat (Iqbal et al., 2021) and tomato (Mukherjee and Bhatla, 2021) plants. The level of H_2S produced by MT could not be high enough in the current investigation to have an adverse impact on plant metabolic processes. When considering our results critically, it is evident that MT can cause the synthesis of H_2S, but pre-treatment with MT and HT also inhibited MT's beneficial effects by scavenging H_2S production.

Arsenate As(V) is absorbed favourably by plants, which mostly store it in their roots and transport a modest quantity to their shoots (Alam et al., 2022). Phosphate transporters also carry As(V), an analog of inorganic phosphate, into plant cells (Sun et al., 2019). Cell membranes, walls (Ender et al., 2019), and phytochelatins (Kiany et al., 2022) prevent the inflow of As into the cell. In our experiment, arsenic was shown to accumulate heavily in plant tissues, especially in the roots as opposed to the upper plant parts (shoots). As was already observed in rice (Ronzan et al., 2019), it caused a greater decrease in root growth than that in the shoot. According to certain studies, H_2O_2 increases metal ions' inflow whereas catalase activity decreases the influx of metal ions (Kaur et al., 2021). Because of this, H_2O_2 increases the permeability of plasma membranes by breaking down lipids and proteins, as demonstrated by decreased membrane stability and APX activity under AsS conditions, thereby allowing more As to enter into the cell (Asgher et al., 2021). The application of MT lowered the subcellular root As in cell wall and organellar fraction, but increased it in soluble fraction in vacuoles. These findings imply that MT functions as a signal, causing absorbed As to be sequestered in vacuoles where it is rendered non-hazardous to other cellular organelles, so its absorption is restricted at the level of the cell wall. Our findings follow the same general pattern as that of Singh et al. (2021) who found that NO sequestered As in vacuoles to neutralize it for other cellular organelles in soybean roots. However, in terms of subcellular As distribution in the leaves during As stress, As was mostly dispersed in the cell wall and cell soluble components. This served a strong evidence that As vacuole compartmentalization and cell wall deposition were the primary causes of pepper's ability to tolerate As stress. It was also observed that after MT treatment, the As content in the pepper leaf cell walls and vacuoles dramatically increased, indicating that MT treatment improved the deposition and fixing of As in the cell walls and vacuoles of pepper leaves. The beneficial impact of MT on As accumulation in the As–S-plants was reversed by HT, which decreased H_2S production. This demonstrates that H_2S might have contributed to MT-induced lowering of As accumulation, which thereby enhance the pepper plants' tolerance to As–S. In pea, the positive impact of the exogenous supply of NaHS (a donor of H_2S), has also been investigated (Alsahli et al., 2021). By inhibiting the As accumulation, the pretreatment of NaHS with MT + HT removed the detrimental impact of HT; this is unquestionably a further proof that H_2S is involved in the rise in As–S tolerance caused by MT. This demonstrates how MT and H_2S both contributed to the reduction of As levels in the As–S pepper plants.

4.4. Oxidative stress is reversed by MT-induced endogenous H_2S

Arsenic exposure predominantly prevents electron transfer during photosynthesis, which results in concomitant oxidative stress (Patel and Parida, 2022). The recognisable signs of oxidative damage, and rises in MDA (lipid peroxidation) and H_2O_2 levels, were both shown to be more prevalent in AsS-plants as compared to the controls. Similar reports indicate that AsS caused oxidative stress, which was associated with elevated MDA and H_2O_2 levels (Zargari et al., 2020; Khan et al., 2022b). Furthermore, Ghorbani et al. (2022) previously reported that elevated MDA and H_2O_2 under As stress enhanced electrolyte leakage in rice. Interrelationships between the attributes have the potential to impair the cell membrane's ability to exchange ions and all related metabolic functions (Siddiqui et al., 2022).

Surprisingly, pre-treatment with MT or MT + NaHS reduced the levels of both H_2O_2 and MDA, thus reversing the effects of oxidative stress on AsS-plants. The protective action of MT against As–S has already been documented in tea (Li et al., 2021) and bean plants (Siddiqui et al., 2022). Since HT treatment lowered the H_2S concentration in As–S plants, it is apparent that H_2S contributes to the beneficial action of MT in reversing oxidative stress. In plants under arsenic exposure, the effect of H_2S in decreasing oxidative stress has been previously studied (Siddiqui et al., 2021; Mishra and Singh, 2021).

4.5. Arsenic in pepper plants is detoxified by MT-induced increased H_2S production

To protect cell organelles from the harmful effects of metal toxicities, such as those of As, phytochelatins (PCs), essential defence compounds, are synthesized from GSH, and chelate toxic metals (Krayem et al., 2022). When PCs attach to As, the complex of PC-As goes to the vacuole and reduces the amount of free As in the cytoplasm (Bhat et al., 2021). Arsenic can also be bound to GSH by the thiol (-SH) group (Kumar and Trivedi, 2018). In order to transform As into a non-toxic state, GSH, a precursor of PCs, can bind to it and transport it to the vacuole (Mostofa et al., 2021). It is possible that As–S-boosted PC synthesis causes As to be detoxified (Singh et al., 2022). Our findings unequivocally demonstrate that PCs and GSH worked in concert to detoxify As in the pepper plants. The GSH levels in the As–S-plants increased, and this GSH then changed into GSSG to detoxify As. The higher GSSG levels in the As-stressed pepper plants are most likely caused by this; As-induced damage to plants is thought to be indicated by higher GSSG concentration and a lower GSH/GSSG ratio (Mishra et al., 2022). The pretreatment of MT raised the GSH/GSSG ratio and GSH content, which reversed the GSH/GSSG ratio and GSH content. The beneficial effects of MT on GSH and GSH/GSSG ratio in As-treated rice plants were also reported by Samanta et al. (2021a).

The As–S-pepper plants produced more PC, and those receiving MT treatment had greater increases in PC levels than those in the control plants. This promisingly shows that MT actively promotes PC production, which in turn triggers the chelation of As to render it harmless. Samanta et al. (2021a) also stated that the chelation of As in rice plants was improved by MT treatment. The PC synthesis was significantly accelerated by MT and NaHS. The combined impact of both substances on the production of PC in As-stressed plants does not appear to have been investigated earlier.

By preventing the synthesis of H_2S in As–S pepper plants, HT pretreatment, however, negated the protective effect of MT on the synthesis of GSH and PC. The removal effect of HT on PC and GSH synthesis under

As–S was inverted by the treatment of NaHS to MT + HT, most likely by reactivating H_2S production. In plants treated with As, the supplemented NaHS-induced increase in PC and GSH production has previously been observed (Singh et al., 2015). This clearly demonstrates how MT and H_2S work together to detoxify As by increasing the production of PC and GSH in As-treated plants.

4.6. Endogenous H_2S produced by MT increases the AsA and GSH levels

Plants generate AsA and GSH, the two most prevalent cellular soluble antioxidants (Gupta et al., 2018), to reinforce the antioxidant defence mechanism and enable plants to respond to ecological challenges (Paul and Roychoudhury, 2020). To sustain the cellular redox balance, the enzymes of antioxidant defence system scavenge ROS (Jung et al., 2020). In comparison to the controls, As–S lowered the reduced form (GSH and AsA) while elevated the oxidised form (GSSG and DHA) in the pepper plants. Oxidative stress in pepper plants brought about by As has a connection to the redox mechanism of AsA and GSH, as reported in rice (Jung et al., 2019; Ghorbani et al., 2021) and mustard (Sahay et al., 2020) plants.

AsA has been found to have a protective role to protect from oxidative stress brought about in stressed plants (Gautam et al., 2021). Furthermore, GSH has been shown to be an efficient scavenger of ROS (Shen et al., 2018), but as was already mentioned, it also gets involved in the production of phytochelatins, which serve as chelators for the detoxification of heavy metals (Mostofa et al., 2021). Through the activation of glutathione-*S*-transferase, GSH also aids in the defence of cell membranes against oxidation of proteins and lipids (Kortheerakul et al., 2021). Our findings, however, showed that As–S increased GSSG and DHA, which resulted in declines in GSH/GSSG and AsA/DHA ratios, thus demonstrating that As stress modifies the oxidation-reduction state of the cell via interaction with the AsA and GSH pools, as earlier reported in rice by Jung et al. (2019) and *Brassica* spp. (Singh et al., 2020a). High GSH/GSSG and AsA/DHA ratios (Pan et al., 2018) show that the pretreatments with MT and MT + NaHS restore the redox status of the AsA and GSH pools. The protective mechanism of the cell against ROS was influenced more by changes in the GSH/GSSG and AsA/DHA ratios than by AsA or GSH concentration alone (Jung et al., 2020). The beneficial effects of MT under AsS on the redox state in our experimental settings are comparable to those observed in cucumber plants exposed to imidacloprid phytotoxicity (Liu et al., 2021). The fact that MT-treated pepper plants retained high GSH and AsA concentrations as well as high GSH/GSSG and AsA/DHA ratios suggests that they might have been more resistant to As stress and may play an important role in shielding these plants from oxidative damage, as was reported in tomato plants exposed to copper toxicity (Zhang et al., 2022). Increased the GSH/GSSG ratio and GSH were shown to be correlated with GR activity induced by MT in the pepper plants under As stress. These results were likewise noted in rice plants treated with MT under As stress (Samanta et al., 2021a), as well as in cucumber plants treated with MT under imidacloprid phytotoxicity (Liu et al., 2021).

The MT's beneficial effects on the elevation of AsA and GSH as well as their redox state during As stress were inhibited by the HT treatment by blocking the production of endogenous H_2S, implying that H_2S is also necessary to increase the MT's capacity for tolerating As stress. As evidenced from the literature no such research has already been conducted illustrating the connection between endogenous H_2S and MT-induced As–S tolerance. However, it has been shown that externally supplied H_2S reinstated the AsA content and AsA/DHA ratio under drought stress in wheat (Shan et al., 2018) and as well as under arsenic stress in tomato (Kaya and Ashraf, 2022). These results show that the restoration of AsA, GSH, and their redox buffer might lead to an improvement in MT-induced As stress tolerance by means of intrinsic H_2S.

4.7. Upregulation of the antioxidant defense system by MT-induced H_2S production

Plants have a built-in antioxidant defence mechanism to prevent excessive ROS generation within cells (Ali et al., 2019). By evaluating the levels of soluble antioxidants and the enzymatic antioxidants' activities, the effect of MT in upregulating the antioxidant defence system has also been evaluated in our study. Upregulation of antioxidant enzymes can increase tolerance to abiotic stresses by bringing ROS levels considerably below the cutoff point (Sachdev et al., 2021).

Superoxide dismutase (SOD), which is regarded as the essential component of defence, plays a significant role in the antioxidant system, by facilitating the catalysis of the dismutation of the $O_2^{\bullet -}$ to H_2O_2 (Mishra and Sharma, 2019). The As-stressed pepper plants showed enhanced SOD activity, comparable to the findings for rice (Wu et al., 2020). When pepper plants were under As stress, their SOD activity was decreased by the pretreatment with MT. These findings coincide with what was shown in spinach (Asif et al., 2020). The induction of SOD, as previously described in rice (Wu et al., 2020), may have been caused by the increased H_2O_2 in the pepper plant leaves treated with As.

The CAT activity in the pepper plants was lowered by arsenic stress, which is in line with what has been shown in rice (Wu et al., 2020). Under As stress, MT-supplied plants displayed increased CAT activity. These results are consistent with what was recorded in spinach (Asif et al., 2020). An effective nitro-oxidative metabolism is naturally present in peroxisomes (Gomaa and Dawood, 2021) and the main peroxisomal antioxidant enzyme, CAT, is known to detoxify H2O2 (Lismont et al., 2019). It has been demonstrated that under the conditions of our experiments, MT treatment boosted the CAT activity and decreased H2O2 levels. It implies that peroxisomes may have contributed to the As–S response mechanism similar to that of lead-induced stress has been documented (Corpas and Barroso, 2017).

Additionally, POD is a crucial enzyme for scavenging H_2O_2, because of its many cellular types (Takács et al., 2018). However, as previously noted in rice (Huang et al., 2018), its activity is recorded to be reduced in plants subjected to As–S. Under As stress, the supply of MT boosted the POD activity in the pepper plants. Asif et al. (2020) and Farooq et al. (2022) previously have shown that MT boosted the POD activity in As-stressed spinach and tea plants, respectively.

The application of HT, which lowered the intrinsic H_2S concentration, reversed the up-regulating impact of MT on the antioxidant enzymes' activities, indicating that endogenous H_2S accumulation contributes to the upregulation of antioxidant defense system induced by MT to increase As–S tolerance of the pepper plants. The supplementation of NaHS with MT under As–S restored the detrimental effects of HT on MT-induced upregulation of the antioxidant enzymes'activities. Earlier studies have demonstrated that antioxidant enzyme activities in bean were increased by exogenously applied H_2S under As–S (Siddiqui et al., 2021). Overall, MT-induced up-regulation of antioxidant enzymes may be associated with pepper plants' tolerance to As stress. As it has been demonstrated that HT treatment decreased CAT and POD activities, but elevated H_2O_2 levels, MT may function well in scavenging ROS via stimulating H_2S production.

4.8. Endogenous H_2S produced by MT stimulates the AsA-GSH cycle related enzymes

The main enzymes implicated in the AsA-GSH pathway, including APX, GR, DHAR, and MDHAR, displayed a considerable shift in their activity in the pepper plants exposed to As–S. Pea seedlings grown under As–S have revealed similar findings (Mishra et al., 2022). Nevertheless, pretreatment with MT along with As (As + MT) considerably mitigated the negative impacts of arsenic-induced decrease in growth indices and photosynthetic characteristics by lowering endogenous As levels, reducing ROS formation, reducing oxidative stress, and increasing the AsA-GSH cycle's constituents. MT alone and in combination with NaHS

may lessen the deposition of arsenic in the leaves and roots by reinstating the function of the AsA-GSH cycle. Additionally, several studies have demonstrated the function of MT in reducing metal absorption and triggering the antioxidant defence system (Goodarzi et al., 2020; Zhang et al., 2022).

One of the key enzymes in the AsA-GSH cycle, which scavenges H_2O_2 through the ascorbate-glutathione cycle, is ascorbate peroxidase (APX), which is mostly present in the plastid stroma and membrane (Dumanović et al., 2021; Jardim-Messeder et al., 2022). As was previously observed in *Isatis cappadocica* (Souri et al., 2020), pea (Alam et al., 2020), and maize (Khan et al., 2022a) plants, the pepper plants under As stress had higher activity of APX in their leaves. Comparable to what was previously observed in rapeseed (Farooq et al., 2022), the pre-treatments of MT and MT + NaHS increased the APX activity in the As-stressed pepper plants. These findings support the argument that H_2S could promote the APX activity in beans grown under As–S (Siddiqui et al., 2021). By removing ROS from stressed plants, glutathione reductase (GR) improves so as to increase resistance to oxidative stress (Wang et al., 2018). As with soybeans (Ahmad et al., 2020), the pepper plants under As stress also displayed increased GR activity in their leaves. In the case of our experiment, The GSH/GSSG ratio and ascorbate pool may have been reduced due to the As-induced increase in GR activity, and had a negative impact on the ascorbate redox state thereby reducing stress tolerance. Under As–S regimes, MT treatment boosted the GR activity, analogous to that previously observed in rapeseed (Farooq et al., 2022).

DHAR is an enzyme that converts DHA into AsA as a part of the AsA-GSH cycle (Das et al., 2022). The DHAR's activity in the leaves of pepper plants was decreased by arsenic stress. Similar outcomes in mustard (Singh et al., 2020b) and rice (Mishra et al., 2022) plants have already been documented. In the current experiment, the pre-treatment of pepper plants with MT under As stress increased the activity of DHAR. Similar findings have been reported in many plant species subjected to various adverse stimuli, such as in *Arabidopsis thaliana* under UV-B exposure (Haskirli et al., 2021) and tomato under water stress (Altaf et al., 2022).

The pepper plants under As stress had a reduction in monodehydroascorbate reductase (MDHAR) comparable to what rice plants have demonstrated (Mishra et al., 2022). The activity of MDHAR in the pepper plants rose as a result of the application of MT, as was previously found in rapeseed (Farooq et al., 2022). MDHAR is essential for maintaining the AsA states and the reduced ascorbate redox pool. It is important to understand that under stressful circumstances, DHAR and MDHAR cooperate to maintain AsA concentration and its redox state (Gomaa and Dawood, 2021).

The activation of the aforementioned AsA-GSH cycle associated enzymes was reversed by the addition of HT, H_2S scavenger, together with MT. This conclusively demonstrates that H_2S activity is necessary to increase MT effectiveness in enhancing the pepper plants' tolerance to arsenic stress. This research appears to be the most important one in the literature, indicating that MT has a function in increasing the H_2S concentration as a downstream signalling molecule of the MT mechanism controlling As tolerance. H_2S has been shown to increase the AsA-GSH cycle when arsenic toxicity is present in pea plants (Alsahli et al., 2021).

4.9. The glyoxalase system is controlled by MT-mediated H_2S production

Under stressful conditions, the glyoxalase system depends on two enzymes, Gly I and Gly II, to prevent an excessive buildup of methylglyoxal (MG) (Alsahli et al., 2021). Comparable to what was shown in rice (Samanta et al., 2021b), MG accumulation increased under arsenic stress conditions. To increase stress tolerance, the over-generated MG must be reversed via the glyoxalase system. Many species may effectively endure abiotic stress, if the activity of the Gly I and Gly II enzymes is upregulated (Hasanuzzaman et al., 2017; Al-Zahrani et al., 2022). According to the results of our experiment, As stress increased the MG content and Gly II activity, while it lowered the Gly I activity. Siddiqui et al. (2022) also noted that As–S enhanced the MG concentration, while it decreased the activities of Gly 1 and Gly 2 in tomatoes. Application of MT significantly boosted both enzyme activities and lowered the MG content, indicating efficient MG elimination and increased As–S tolerace. MT-induced reduction in MG accumulation and elevation in the glyoxalase system related enzymes have been reported in rice (Samanta et al., 2021b).

The supply of HT inverted the positive impact of MT on the glyoxalase system and detoxifying effect of MG by preventing the synthesis/accumulation of H_2S in the pepper plants under AsS, indicating that H_2S is required for both the MT-induced activation of the glyoxalase system and the MT-induced detoxification of MG. Application of NaHS along with MT upturned the reversing effect of HT on the positive effect of MT to detoxify MG and upregulate the glyoxalase system in the pepper plants under As–S. The role of exogenously administered H_2S in enhancing the glyoxalase system's effectiveness has previously been observed in pea (Alsahli et al., 2021).

5. Conclusions

The exogenous administration of MT together with H_2S helped alleviate oxidative stress generated by arsenate in pepper plants by simultaneously upregulating the antioxidant and glyoxalase systems. Large-scale field experiments and other research projects similar to the current one might be helpful in creating a sustainable strategy for food production, particularly in arsenic-contaminated areas. MT and H_2S work together to regulate cellular As accumulation in the vacuoles of roots and leaves, thereby restricting its mobility to cell organelles. The information presented here also opens up new directions for fundamental studies on the interaction of MT and H_2S, and how they act at the molecular level to regulate both the AsA-GSH and the glyoxalase systems.

Statement of authorship

Both the experiment and data analysis were done by CK and FU. MNA and AB analyzed the data and helped in statistical analysis. The first draft of the text was also written by CK and FU. MA and PA contributed to the study's planning and thoroughly edited the finished version. The final manuscript was reviewed and approved by all authors.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgement

The author wishes to thank the University of Harran (Turkey) for partly supporting this study. The authors would also like to extend their sincere appreciation to the Researchers Supporting Project Number (RSP-2021/180), King Saud University, Riyadh, Saudi Arabia.

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