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RESEARCH PAPER

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DUAL ROLE OF HYDROGEN SULFIDE IN MODULATING PHOTOSYNTHESIS, ANTIOXIDANT DEFENSE, AND MEMBRANE INTEGRITY IN *Cucurbita pepo*

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ABSTRACT

Although traditionally regarded as a toxic environmental gas, hydrogen sulfide (H_2S) has recently been recognized as a gasotransmitter involved in regulating various physiological processes in both plants and animals. This study aimed to investigate the stage- and concentration-dependent effects of exogenous H_2S on the growth, photosynthetic capacity, and antioxidant performance of squash (*Cucurbita pepo*) plants. Fifteen-day-old seedlings were subjected to foliar application of H_2S at different concentrations (0, 25, 50, 75, 100, 200, and 300 µM) and monitored until the end of the experiment. Plant samples were collected at two distinct intervals following H_2S treatment – 24 hours and 15 days to comprehensively assess growth, physiological, and biochemical parameters. The results revealed a biphasic response to H_2S treatments. Application of 100 µM H_2S significantly improved growth traits (including shoot and root length, dry biomass, and leaf area), photosynthetic performance, carbonic anhydrase activity, antioxidant enzyme activities, and proline accumulation, while reducing electrolyte leakage and lipid peroxidation compared to untreated controls. In contrast, higher concentrations (200 and 300 µM) adversely affected these parameters and caused increased cellular damage. These findings suggest that 100 µM H_2S is the optimal concentration for enhancing physiological and biochemical traits in squash and may serve as a promising tool for improving crop productivity via improved photosynthetic and stress-response mechanisms.

Keywords: antioxidants, hydrogen sulfide, photosynthesis, proline

INTRODUCTION

Historically, hydrogen sulfide (H_2S) has been regarded as a toxic environmental gas characterized by its pungent odor resembling rotten eggs. It is an inorganic, water-soluble gas that, at high concentrations, poses toxicity risks to living organisms. However, over the past two decades, H_2S has emerged as a crucial gasotransmitter, a signaling molecule analogous to nitric oxide (NO), carbon monoxide (CO), and hydrogen peroxide (H_2O_2) that regulates a wide range of physiological processes in both plants and animals [Vandiver and Snyder 2012, Kimura 2014, Aroca et al. 2018]. In plants, H_2S is biosynthesized via two main

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pathways: (i) the sulfate assimilation route, where sulfate is reduced to sulfide through a series of enzymatic reactions involving ATP sulfurylase, APS reductase, and sulfite reductase, and (ii) the cysteine-dependent pathway, where enzymes such as L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD) catalyze the conversion of cysteine to H_2S [Alvarez et al. 2010, Li et al. 2016, Hancock and Whiteman 2016]. These biosynthetic processes occur in subcellular compartments including chloroplasts, mitochondria, and the cytosol [Corpas et al. 2019].

Recent studies have established the pivotal roles of H_2S in regulating seed germination, root architecture, photosynthesis, and organogenesis, both under optimal and stress conditions [Arif et al. 2021, Zhou et al. 2021]. At low concentrations, H_2S enhances chlorophyll synthesis, boosts the activity of photosynthetic enzymes like ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), and promotes the formation of functional chloroplasts [Xie et al. 2014, Ye et al. 2020]. In contrast, high concentrations of H_2S have been shown to exert inhibitory effects, leading to growth retardation and oxidative stress, thereby confirming its concentration-dependent duality [Khan et al. 2017, Liu et al. 2019, Corpas and Palma 2020]. Additionally, H_2S plays an active role in activating both enzymatic and non-enzymatic antioxidant defense mechanisms, reduces lipid peroxidation, and facilitates osmoprotection by enhancing proline accumulation and carbonic anhydrase activity under abiotic stress [Khan et al. 2017, Zhang et al. 2022]. H_2S is also a key player in the complex signaling network of plants, exhibiting crosstalk with various phytohormones and signaling molecules. It interacts synergistically or antagonistically with abscisic acid (ABA), gibberellic acid (GA), and ethylene to regulate stomatal movement, senescence, and stress adaptation [Liu et al. 2012, Scuffi et al. 2014, Xie et al. 2014]. Furthermore, H_2S is known to modulate NO and H_2O_2 signaling, thus influencing redox balance and cellular homeostasis [Zhang et al. 2010, Lisjak et al. 2011]. Despite these advances, many mechanistic aspects surrounding H_2S signaling, including its precise biochemical regulation and interactions with other pathways, remain underexplored and are active areas of research [Aroca et al. 2018, Corpas et al. 2019].

Given these multifaceted roles, H_2S has gained attention as a promising agent for enhancing plant resilience and productivity. The present study was therefore designed to investigate the stage- and concentration-dependent effects of exogenously applied H_2S on squash (*Cucurbita pepo*) plants. Specifically, the research aims to explore how different H_2S concentrations influence plant growth, photosynthetic performance, and the modulation of enzymatic and non-enzymatic antioxidant defense systems, thereby identifying optimal conditions for potential agricultural application.

MATERIALS AND METHODS

Biological material

Squash (*Cucurbita*) seeds were procured from a local seed market, Al Ain, UAE. Healthy and uniformly sized seeds were washed 2–3 times with deionized water and then sterilized with 0.01% mercuric chloride ($HgCl_2$) aqueous solution followed by repeated washing with deionized water.

Preparation of hydrogen sulfide

Sodium hydrosulfide (NaHS) was used as the donor compound for H_2S. A 1 mM stock solution was prepared by dissolving the appropriate amount of NaHS in a small volume of deionized water and adjusting the final volume to 100 mL with deionized water. Working solutions with final concentrations of 0, 25, 50, 75, 100, 200, and 300 µM H_2S were prepared by serial dilution of the stock solution. Prior to foliar application, a surfactant (Tween-20) was added to the solutions to enhance leaf surface absorption.

Experimental set up and treatment patterns

Thirty-five plastic pots (23 cm in diameter) were filled with commercial potting soil and placed in a plant growth chamber under controlled environmental conditions (temp: 25 °C day /20 °C night; light: 14−16 h photoperiod, 400–600 PPFD; RH: 60–70%; airflow: constant gentle circulation). Five replicate pots were assigned to each of the seven treatments and arranged in a completely randomized block design. Surface-sterilized squash seeds were sown in each pot and allowed to germinate. When germination began, the seedlings were thinned to maintain three plants per pot. At 15 days after sowing, the plants were subjected to a foliar application

of H_2S at concentrations of 0 (deionized water, control), 25, 50, 75, 100, 200, or 300 µM. Each plant was sprayed three times with its respective H_2S solution using a sprayer nozzle adjusted to deliver approximately 1 mL per spray. The plants were grown until 30 days after sowing. Plant samples were collected at two time points: 16 days after sowing (24 hours after H_2S treatment) and 30 days after sowing. These samples were analyzed for various growth, physiological, and biochemical parameters.

Assessment of plant growth biomarkers

The plants from each replicate were gently uprooted and thoroughly washed with tap water to remove adhering soil particles. After washing, roots and shoots were separated using a scalpel, and their respective lengths were measured. The separated roots and shoots were then placed in a hot air oven at 70 °C for dehydration. Upon complete drying, the dry weights of both roots and shoots were recorded.

Leaf area was determined using a gravimetric method. Leaves were randomly selected from each treatment group, and their outlines were traced onto graph paper to calculate the area.

Determination of leaf relative water content (LRWC)

To determine leaf relative water content (RWC) we weighed fresh leaf discs of 2 cm diameter, excluding midrib, and floated discs on deionized water in Petri dishes for 24 hours in the dark, where they remained saturated with water. The adhering water on the discs was blotted out, and the turgor mass was measured. The discs were dehydrated at 60 °C for 72 h, and the LRWC was calculated as:

$$LRWC = (FM – DM) / (TM – DM) \times 100$$

where: FM = fresh mass; DM = dry mass; TM = turgor mass.

Chlorophyll content

Using the method of Arnon (1949), 1 g of fresh leaves were homogenized in a mortar with sufficient amounts of 80% acetone. The chlorophyll extract was placed in a centrifuge tube and diluted with 80% acetone to make 10 mL. The supernatant was collected in a cuvette and absorbance was measured at 645 nm and 663 nm on a spectrophotometer.

Determination of net photosynthetic rate and stomatal conductance

An infrared gas analyzer (IRGA) portable photosynthetic system (LI-COR 6400, LI-COR, Lincoln, NE, USA) was used to measure gas net photosynthetic rate and stomatal conductance on the third fully expanded leaves between 11.00 and 12.00 h. Air temperatures, relative humidity, CO_2 concentration, and PPFD were adjusted to 25 °C, 85%, 600 µmol mol^{-1} and 800 µmol mol^{-2} s^{-1} to measure net photosynthetic rate and stomatal conductance, respectively.

Measurement of carbonic anhydrase (CA) activity

Carbonic anhydrase (CA) activity in fresh leaves was assessed using the method described by Dwivedi and Randhawa [1974]. Fresh leaves from each replicate were cut into small pieces, and 200 mg of these pieces were weighed and transferred to Petri plates containing 10 mL of cysteine hydrochloride solution. The samples were incubated at 4 °C for 20 minutes. After incubation, the leaf pieces were blotted dry and transferred to fresh tubes containing phosphate buffer (pH 6.8). Each tube was then supplemented with 4 mL of sodium bicarbonate solution and 0.2 mL of bromothymol blue. The tubes were shaken and incubated again at 4 °C for 20 min to allow the catalytic action of carbonic anhydrase on $NaHCO_3$, resulting in the liberation of CO_2. The amount of CO_2 released was estimated by titrating the reaction mixture against 0.05 N HCl using methyl red as an indicator. A blank, containing all components of the reaction mixture except the leaf sample, was run in parallel with each set of samples.

Determination of leaf electrolyte leakage and lipid peroxidation

Leaf electrolyte leakage was calculated from the total inorganic ions leaked out from the leaves by the method described by Sullivan and Ross [1979].

According to Hodges et al. [1999], the malondialdehyde equivalents of the leaf were determined by homogenizing the leaves in 80% ethanol and centrifuging them at 3000 g for 10 min at 4 °C.

The pellet obtained after centrifugation was extracted twice with the same solvent, and the resulting supernatants were pooled in a test tube. In the same test tube, butylated hydroxytoluene (0.15%) and thiobarbituric acid (0.65%) were simultaneously added along

with supernatants. The reaction mixture was prepared by combining equal volumes of the supernatant, 20% trichloroacetic acid, 0.01% butylated hydroxytoluene, and 0.65% thiobarbituric acid . The mixture was then heated at 95 °C for 25 min and subsequently cooled to room temperature. Optical density of sample was measured at 440, 532, and 600 nm and using these values, rate of lipid peroxidation was calculated according to the formula given by Hodges et al. [1999].

Analysis of antioxidant enzymes (catalase, peroxidase, and superoxide dismutase)

Fresh leaves (1 g) were homogenized with cold lysis buffer (70 mM phosphate buffer; pH 7.0, 1 mM EDTA, 1 mM PMSF, 0.5% Triton X-100 and 2% PVP) and homogenate was centrifuged at 12000 × g for 20 min at 4 °C. Supernatant was collected and stored at –20 °C for the analysis of catalase, peroxidase, and superoxide dismutase activities.

Catalase activity was determined utilizing the method of Aebi [1984]. Reaction mixture (50 mM phosphate buffer – pH 7.0, 15 mM H_2O_2 and 100 µL enzyme extract) was prepared to measure the loss of H_2O_2 during the start of the reaction with the help of a spectrophotometer at 240 nm for 2 min.

For the analysis of peroxidase activity, method of Sánchez et al. [1995] with slight changes was performed. In this method, reaction mixture with 50 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 1.5 mM H_2O_2 and 100 µL enzyme extract was prepared. The enzyme activity in reaction mixture was measured as absorbance at 436 nm for 1 min at 25 °C.

During the estimation of superoxide dismutase activity, a reaction mixture was prepared using 50 mM phosphate buffer (pH 7.8), 9.9 mM L-methionine, 55 µM nitroblue tetrazolium (NBT), 2 mM EDTA, 0.02% Triton X-100, 40 µL enzyme extract and 1 mM riboflavin (added at last). The absorbance of sample prepared was measured at 560 nm for 2 min at 25 °C.

Determination of proline content

Using the Bates et al. [1973] method, fresh leaf samples were extracted in sulphosalicylic acid, followed by additions of acetic acid and ninhydrin solutions. A 5 mL solution of toluene was subsequently added to the extract, at 100 °C, together with equal volumes of glacial acetic acid and ninhydrin solutions. The reaction was terminated in ice bath and absorbance of chromophore (toluene layer) recorded at 528 nm on a spectrophotometer.

Statistical analysis

Graphs presented in manuscript are the mean of five replicates (n = 5) ±standard error. Data were statistically analyzed with the help of ANOVA and Tukey's HSD test was used to determine significant different between means at $p \leq 0.05$ level using Minitab 17, Statistical software, UK.

RESULTS

Growth performance

As shown in Figure 1, the application of H_2S at various concentrations (25–300 µM) had differential effects on key growth parameters such as shoot and root length, dry mass, and leaf area at two different growth stages i.e., 1- and 10-day post-treatment. No significant changes were observed at 1 day after treatment. However, at 15 days post-treatment (30 days after sowing), 100 µM H_2S significantly enhanced all growth indices compared to the control and other concentrations. In contrast, higher concentrations (200 and 300 µM) exhibited phytotoxic effects and significantly reduced growth parameters relative to untreated plants.

Leaf relative water content (RWC)

H_2S treatments elicited notable shifts in leaf RWC at both 15 and 30 days after sowing (Fig. 2A). While lower concentrations (≤100 µM) either maintained or improved RWC, the 100 µM treatment showed the most pronounced effect, increasing RWC by 21.5% at 30 days post-sowing compared to control plants. Conversely, high concentrations (200 and 300 µM) significantly reduced RWC, with more severe effects at the later growth stage.

Chlorophyll content

The chlorophyll content responded positively to 100 µM H_2S, which induced significant increases of 37.3% and 29% at 16 and 30 days after sowing, respectively (Fig. 2B). Lower concentrations (25 and

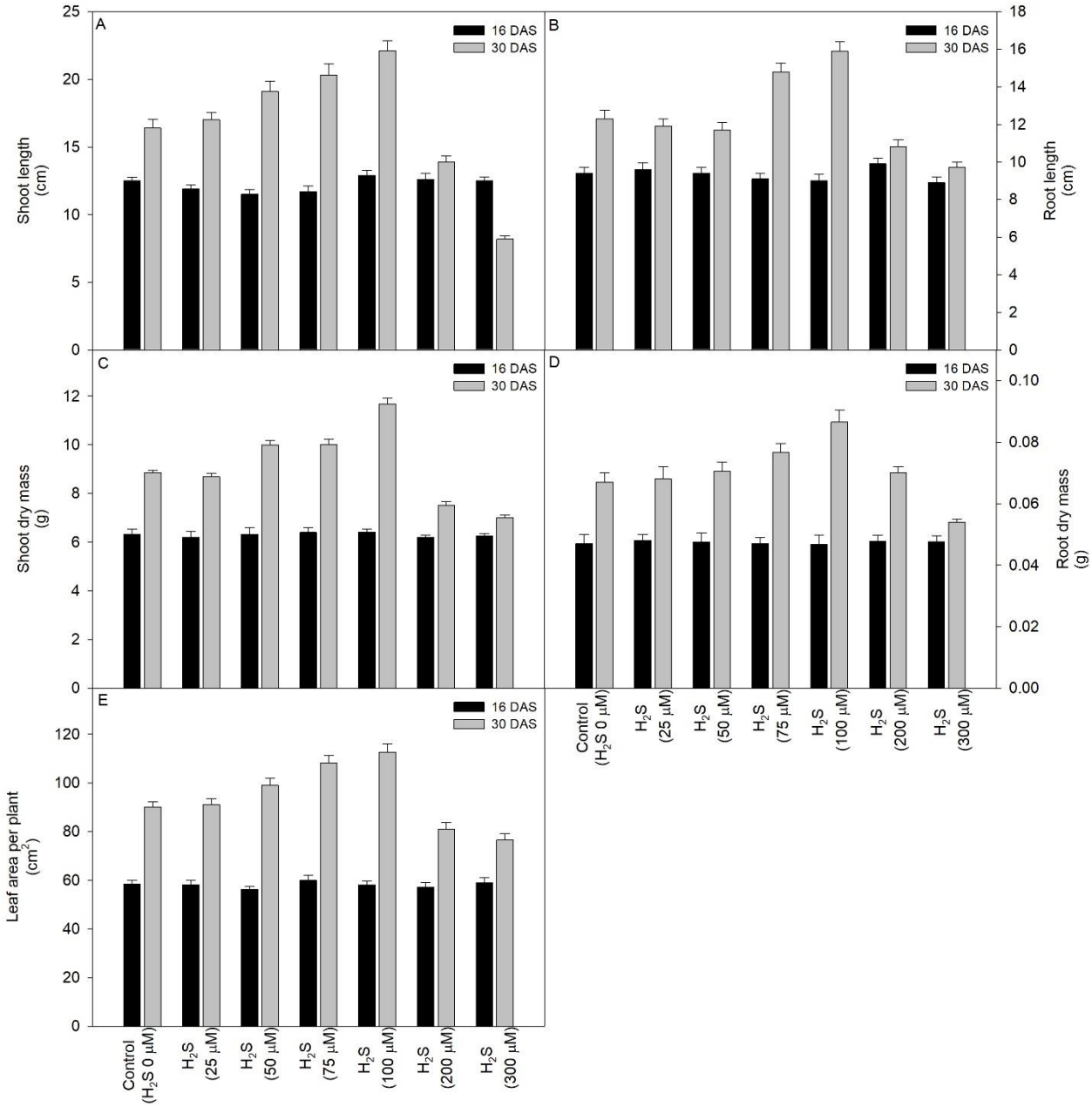

Fig 1. Hydrogen sulfide (H_2S) at concentrations of 0, 25, 50, 75, 100, 200, and 300 µM induced changes in (A) shoot length, (B) root length, (C) shoot dry mass, (D) root dry mass, and (E) leaf area of *Cucurbita* (squash) plants, measured at 16 and 30 days after sowing (DAS). Data represents the mean of five replicates (n = 5), and vertical bars indicate standard errors (±SE)

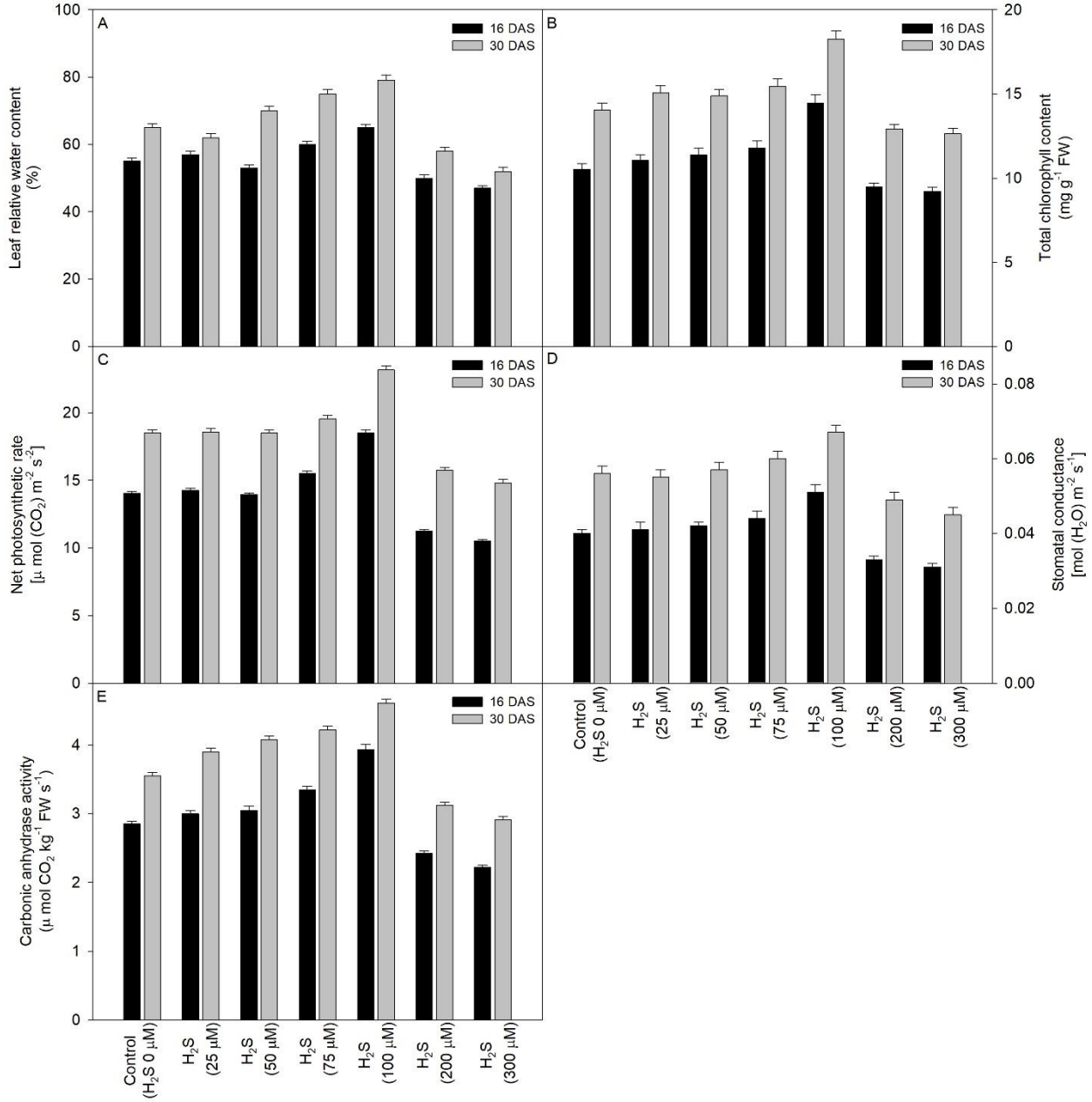

Fig 2. Hydrogen sulfide (H_2S) at concentrations of 0, 25, 50, 75, 100, 200, and 300 µM induced changes in (A) leaf relative water content, (B) total chlorophyll content, (C) net photosynthetic rate, (D) stomatal conductance, and (E) carbonic anhydrase activity of *Cucurbita* (squash) plants, measured at 16 and 30 days after sowing (DAS). Data represents the mean of five replicates (n = 5), and vertical bars indicate standard errors (±SE)

50 μM) had negligible effects, while 200 and 300 μM treatments led to marked chlorophyll degradation, indicating potential oxidative or metabolic stress at higher doses.

Net photosynthetic rate and stomatal conductance

Photosynthetic rate and stomatal conductance (Figs 2C and 2D) increased significantly in response to increasing H_2S concentrations up to 100 μM. Maximum enhancement was observed at 100 μM, beyond which (at 200 and 300 μM), both parameters declined sharply. Notably, the 300 μM treatment led to reductions of 25% and 20% at 16 and 30 days after sowing, respectively, underscoring a dose-dependent biphasic response.

Carbonic anhydrase activity

Carbonic anhydrase activity exhibited a similar biphasic pattern (Fig. 2E). The 100 μM H_2S treatment enhanced enzyme activity by 37.8% and 29.5% at 16 and 30 days after sowing, respectively, relative to control plants. While low concentrations had no notable effect, higher doses (200 and 300 μM) significantly suppressed enzyme activity, with the impact more pronounced at the earlier stage.

Membrane integrity: electrolyte leakage and lipid peroxidation. Membrane stability, assessed via electrolyte leakage and lipid peroxidation, was adversely affected at high H_2S concentrations (200 and 300 μM), indicating oxidative stress (Fig. 3). In contrast, 100 μM H_2S minimized both parameters at both growth stages, suggesting a protective effect at this concentration. Lower concentrations had a negligible influence.

Antioxidant enzyme activities (CAT, POX, and SOD). Treatment with H_2S modulated the activities of key antioxidant enzymes in a concentration-dependent manner. CAT activity increased with concentration up to 100 μM, peaking with enhancements of 62.2% and 50% at 16 and 30 days post-sowing, respectively (Fig. 4A). Higher concentrations reduced CAT activity, indicating enzyme inhibition under stress.

POD activity progressively increased with concentration (Fig. 4B), with maximum elevations of 74.1% and 47.2% at 100 μM at 16 and 30 days after sowing, respectively.

SOD activity revealed that 100 μM H_2S consistently promoted SOD activity at both time points, while other concentrations showed variable trends (Fig. 4C), reflecting differential redox modulation.

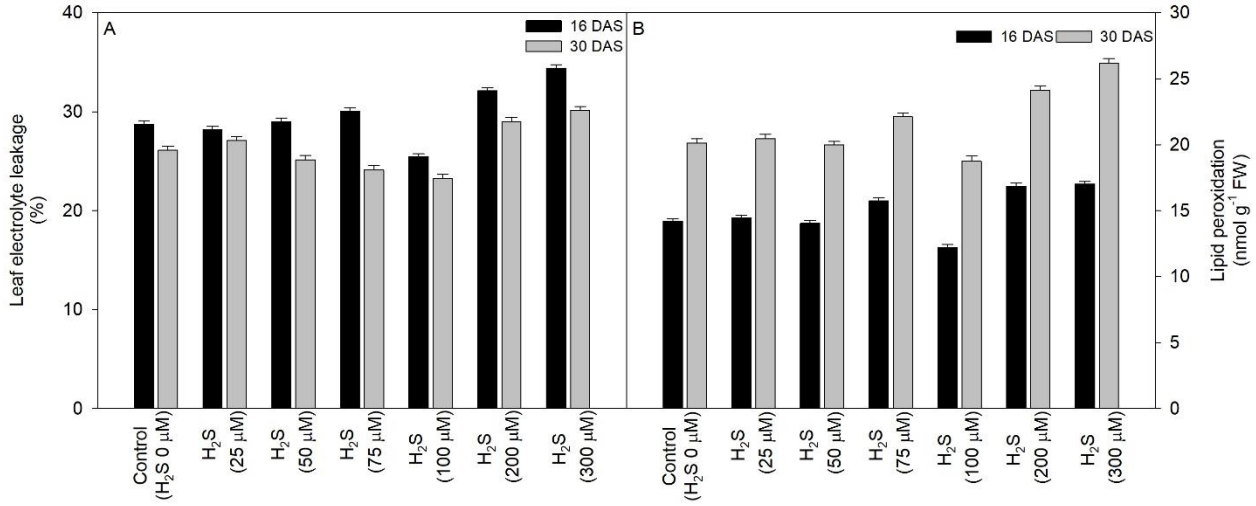

Fig 3. Hydrogen sulfide (H_2S) at concentrations of 0, 25, 50, 75, 100, 200, and 300 μM induced changes in (A) leaf electrolyte leakage, and (B) lipid peroxidation of *Cucurbita* (squash) plants, measured at 16 and 30 days after sowing (DAS). Data represents the mean of five replicates (n = 5), and vertical bars indicate standard errors (±SE)

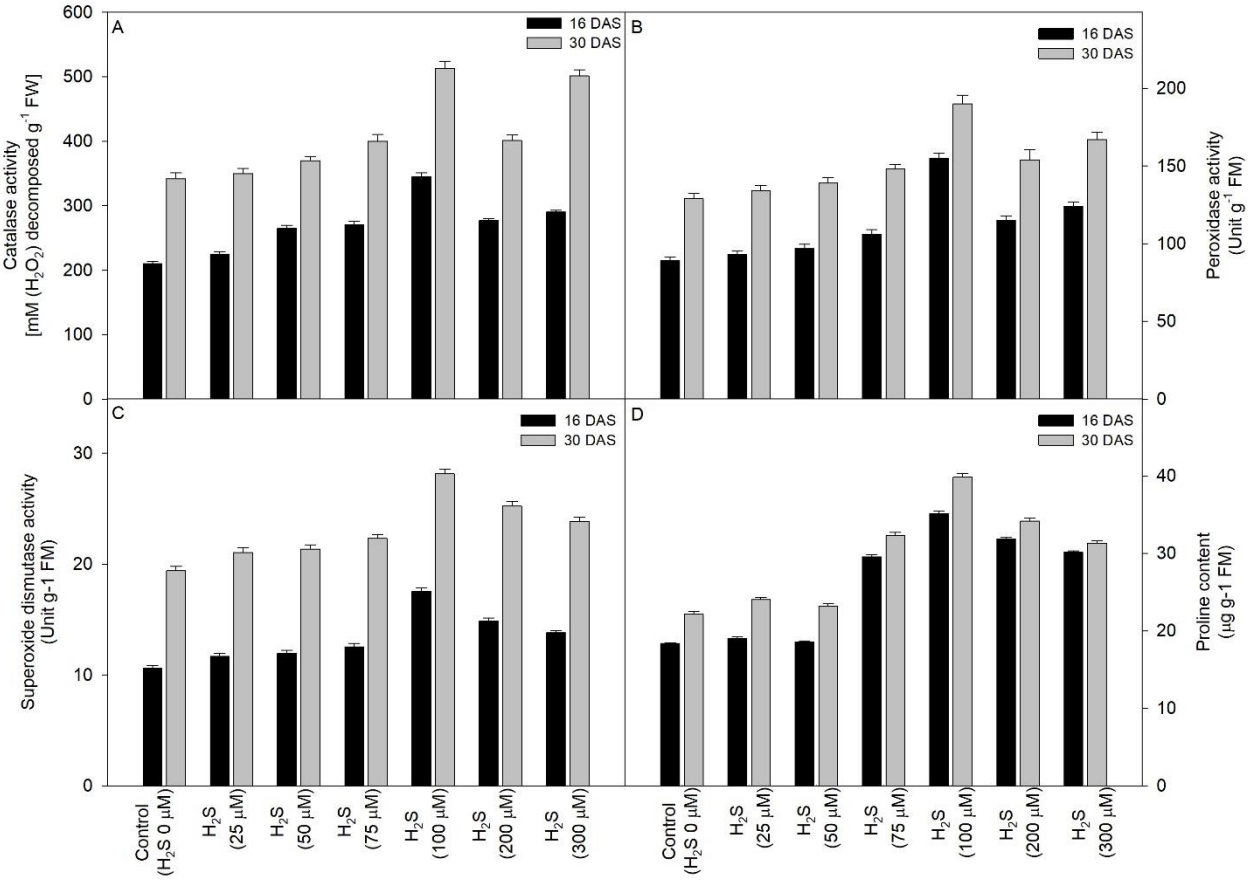

Fig 4. Hydrogen sulfide (H_2S) at concentrations of 0, 25, 50, 75, 100, 200, and 300 µM induced changes in activities of (A) catalase, (B) peroxidase, (C) superoxide dismutase, and (D) proline content of *Cucurbita* (squash) plants, measured at 16 and 30 days after sowing (DAS). Data represents the mean of five replicates (n = 5), and vertical bars indicate standard errors (±SE)

Proline accumulation. Proline content, a marker of osmotic stress response, was significantly elevated by 100 µM H_2S (Fig. 4D), with increases of 64.9% and 44.9% at 16 and 30 days after sowing, respectively, over control. Lower concentrations had minimal effects, while higher doses were less effective, suggesting an optimal concentration window for osmoprotection.

DISCUSSION

The present study demonstrates the concentration-dependent effects of exogenous H_2S on squash (*Cucurbita pepo*) seedlings, revealing its dual role as both a growth promoter and a potential phytotoxin and shows involvement in the production of ROS in plants [Zhang et al. 2017]. The application of 100 µM H_2S emerged as the most effective treatment, significantly enhancing growth attributes such as shoot and root length, dry biomass, and leaf area. These findings are consistent with recent reports indicating that low concentrations of H_2S can stimulate plant growth through improved nutrient assimilation, hormonal regulation, and photosynthetic performance [Alamer 2023, Guo et al. 2023]. Notably, 100 µM H_2S also enhanced chlorophyll content and photosynthetic rate, supporting the role of H_2S in modulating chloroplast function and carbon assimilation under optimal concentrations [Chen et al. 2011].

Improvement in physiological parameters such as RWC and reduced membrane damage as indicated by lower electrolyte leakage and lipid peroxidation under 100 μM H_2S treatment suggests enhanced water retention and membrane stability, likely through modulation of osmotic and oxidative stress pathways. This aligns with previous findings where H_2S treatment improved drought and salinity tolerance by enhancing cell turgor and reducing oxidative damage [Christou et al. 2014, Mostofa et al. 2015]. The significant accumulation of proline in treated plants further supports the hypothesis that H_2S mediates osmoprotection by modulating osmolyte biosynthesis and stress signaling [Hao et al. 2020, Mingjian et al. 2025]. At the enzymatic level, the upregulation of antioxidant enzymes such as catalase, peroxidase, and superoxide dismutase under 100 μM H_2S indicates a robust antioxidative defense mechanism activated by H_2S to scavenge excess ROS. This response reflects the protective role of H_2S in maintaining redox homeostasis under environmental stress conditions, as previously reported in wheat, rice, and *Arabidopsis* models [Wang et al. 2022, Du et al. 2021, Jurado-Flores et al. 2023]. Moreover, recent mechanistic insights suggest that H_2S may regulate these antioxidant pathways through post-translational modifications such as protein persulfidation, thereby altering the activity and stability of redox-related proteins [Aroca et al. 2018, Huang et al. 2021].

However, the adverse effects observed at higher concentrations (200 and 300 μM), including suppressed growth, reduced chlorophyll content, and impaired antioxidant responses, underline the potential toxicity of excessive H_2S. This biphasic effect emphasizes the importance of dosage, as high levels of H_2S can trigger oxidative stress rather than alleviate it, possibly due to overaccumulation of sulfur-containing metabolites or disruption of homeostasis [Li et al. 2013, Huang and Xie 2023]. Such findings are in agreement with earlier reports that highlighted the hormonic behavior of gaseous signaling molecules like H_2S, where a narrow concentration window determines beneficial versus detrimental outcomes [Daneshvand et al. 2024].

CONCLUSION

It is concluded that squash plants exhibit stage-specific and concentration-dependent responses to exogenous H_2S application. Among the tested concentrations (25, 50, 75, 100, 200, and 300 μM), the higher doses (200 and 300 μM) induced deleterious effects at both developmental stages, with more pronounced damage observed at the early growth stage. In contrast, 100 μM H_2S consistently enhanced photosynthetic efficiency and antioxidant activity at both stages, while significant improvements in growth-related traits were particularly evident at 30 days after sowing. Therefore, 100 μM H_2S appears to be the optimal concentration for improving physiological and biochemical performance in squash, offering potential for enhancing crop productivity through improved photosynthetic capacity. However, caution must be exercised in its application, as supra-optimal doses can reverse these benefits. These insights contribute to a growing body of evidence advocating for the strategic use of gasotransmitters like H_2S in sustainable agriculture to bolster crop resilience under abiotic stress conditions.

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CONFLICT OF INTEREST

The author declares that they have no conflict of interest.

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