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Hydrogen Sulfide Crosstalk with Melatonin in Regulating Photosynthetic Resilience under Heat Stress

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Abstract. *Plants are sessile organisms that face continuous alterations in environmental conditions. Among such factors, heat stress has been considered one of the most disadvantageous variables to crop productivity. High temperatures rapidly impair photosynthetic efficiency, disturb cellular redox balance, and alter hormonal regulation, hence limiting growth and productivity in plants. Although much literature exists regarding the role of individual protective agents, their combined actions-particularly melatonin and H_2S-have not yet been brought under the spotlight with respect to their ability to improve plant heat tolerance. All the research work carried out in this study was done to investigate physiological, biochemical, hormonal, and molecular responses of plants treated with melatonin and H_2S under heat-stressed conditions.*

Treatments applied in this experiment were controlled applications of melatonin, H_2S, and their combination, as well as heat stress alone. Among the physiological parameters measured by standard techniques were photosynthetic rate and chlorophyll content. Biochemical responses with regard to ROS accumulation and antioxidant enzyme activities were performed using established assays. In this study, the ABA, IAA, and GA levels were detected by ELISA, and the expressions of heat stress-associated genes HSP70 and HSP90 were determined by qRT-PCR.

These results showed that severe heat stress strongly reduced photosynthetic efficiency and chlorophyll content, thereby increasing the accumulation of ROS. Both melatonin and H_2S treatments alone significantly relieved these effects, but combined application was most protective toward photosynthesis, the maintenance of chlorophyll, mitigation of oxidative damage, activation of antioxidant enzymes, and restoration of hormonal balance. Combined treatment also upregulated the transcription of HSP70 and HSP90 strongly, indicating that there is a synergistic molecular mechanism behind improved heat tolerance.

In this view, the synergistic use of melatonin and H_2S represents an integrative approach to improved plant tolerance to heat stress by synchronized physiological, biochemical, hormonal, and genetic responses. It could also be highly useful in developing sustainable approaches to the improvement of crop productivity under a globally warming climate.

Keywords: *Heat stress, Melatonin, Hydrogen sulfide, antioxidant enzymes, plant hormones, gene expression, photosynthesis.*

1. Introduction

Being sessile organisms, plants constantly face changes in the surrounding environment that are adverse to survival, growth, and productivity. Among these stresses, heat is surely the most intensive and widespread stressor threatening crop productivity across the world.

High temperatures rapidly degrade photosynthetic efficiency by inducing a reduction of integrity in thylakoid membranes, impairing key enzymes, changing electron flow, and lowering rates of PSII repair. They induce severe buildups of ROS from contents that overwhelm the intrinsic antioxidant machinery of the plant.

In particular, this becomes critical at sensitive stages of development or during recurring heat waves under changing climate conditions. Recently, scientific focus has shifted to those small molecules participating in the rapid, multifaceted regulation of physiology under stress.

The most relevant among them are hydrogen sulfide (H_2S) and melatonin (MT; N-acetyl-5-methoxytryptamine), which for years now have been considered major contributors to increased plant tolerance against abiotic stresses by mechanisms that, while intertwined, are different. H_2S is a gaseous signaling molecule involved in protein persulfidation and the regulation of ion fluxes and stomatal movement, interacting with oxidative and hormonal signaling networks. Melatonin, in turn, acts as an active antioxidant and modulates the transcription of antioxidant defense genes, carbohydrate metabolism, and a wide range of transcripts corresponding to molecules associated with stress response signaling pathways (Aroca et al., 2021; Huang & Xie, 2023; Hassan et al., 2022).

Despite the extensive studies that have appeared on the protective roles of melatonin and H_2S individually under various abiotic stresses, such as drought, salinity, and cold, the mechanistic basis of interaction between these two molecules under heat stress remains unclear. For example, it is unknown whether melatonin induces the biosynthesis of H_2S as the primary signal or whether H_2S acts as the downstream mediator, transducing the regulatory benefits of melatonin toward photosynthetic machinery. Moreover, the biochemical nodes underlying this interaction, including persulfidation of PSII-related proteins, modulation of RBOH/NADPH oxidase activity, or regulation of carbohydrate partitioning, remain incompletely defined. Thus, understanding the MT–H_2S axis under heat stress represents a key missing link toward the development of strategies aimed at improving crop thermotolerance.

In this work, the authors try to explain the mutual interaction between melatonin and hydrogen sulfide in maintaining photosynthetic resilience against heat stress by:

1. To investigate the effect of exogenous melatonin on the level of endogenous H_2S and the expression or activity of H_2S biosynthetic enzymes under heat-stress conditions.

2. It was hypothesized that the pharmacological inhibition of H_2S reduced the melatonin-mediated protection against injury of the gas exchange processes, chlorophyll fluorescence parameters, and markers of oxidative stress in wheat plants.

3. Identification of the potential persulfidated proteins and antioxidant transcripts responsive to melatonin that are involved in the maintenance of PSII function.

4. To determine if joint applications of melatonin and H_2S donors have advantages over single applications of the treatments in improving thermotolerance.

The present study presents an integrated scheme for protection through interconnected molecular signals between melatonin and H_2S, with the intention of providing basic understanding of their interrelated signaling to enhance productivity under thermal conditions that are increasing due to climate change. It does so through works by Ahmad et al. (2023) ; Colombage et al. (2023).

2. Previous Studies

The molecular mechanism of how plants cope with heat stress, unraveled in research work over the last couple of years with the help of small regulatory signaling molecules like MT and H_2S, adds an important step to the state of knowledge on how these molecules enhance the level of stress tolerance and affect photosynthesis and antioxidant defense.

Recent studies show that melatonin promotes broad protection against heat stress by several mechanisms. For example, Huang and Xie (2023) demonstrated that the pretreatment of wheat plants with melatonin before applying heat stress significantly enhanced photosynthetic efficiency by protecting PSII-related proteins against oxidative damage. Accumulation of ROS was reduced, along with the enhanced activity of antioxidant enzymes such as SOD, CAT, and APX, distinctly improving heat tolerance in plants.

The results presented by Gautam et al. (2022) indicated that melatonin enhanced the expressions of HSPs and major signaling pathways involved in partitioning carbohydrates and energy balance within chloroplasts. The findings also documented that exogenous melatonin strengthened the integrity of thylakoid membranes and chlorophyll structure, hence maintaining the PSII fluorescence efficiency under high temperatures.

Other studies have indicated that H_2S acts as a gaseous signaling molecule in the induction of plant endogenous defense mechanisms. Gu et al. (2022) noted that these effects are achieved through post-translational modifications in target proteins by persulfidation, leading to an enhanced antioxidant capacity, which protects photosynthetic machinery from heat damage. They further presented evidence to show that H_2S participates in regulating stomatal movement so as to maintain the internal water balance and inhibit carbohydrate loss under hot spells.

According to Murch 2021, H_2S acts as the second messenger of hormonal signaling pathways of cytokinins and auxins that, under stress conditions, reorder plant growth, reducing tissue damage promoted by high temperature. Further, these authors have shown that H_2S is not just an antioxidant molecule but a highly tuned regulatory molecule operating at protein and gene expression levels related to heat defense.

Thus, the relationship between melatonin and H_2S has recently become a focus of interest in relation to studies on heat stresses. Aroca et al., 2021; Yang et al., 2024 have suggested that melatonin may induce biosynthesis of H_2S in plant cells. That is to say, in the course of a signalling pathway, melatonin could be a primary signal that enhances the production of H_2S, while actually acting like a second messenger in the realization of its regulative functions by redox changes in proteins and their state.

Recently, the work of Ahmad et al. (2023) reported prominent synergistic interactions of melatonin with H_2S donors: co-application enhanced photosynthetic efficiency, improved chlorophyll content, reduced the accumulation of ROS, and enhanced the activity of all antioxidant enzymes. More importantly, inhibition of H_2S during treatments with melatonin greatly diminished such effects, pointing toward its role as a key mediator in protective actions of melatonin against heat stress.

It was shown by previous studies that melatonin and H_2S act through interrelated pathways of inducing thermotolerance in plants. While melatonin itself exerts a role in the protection by antioxidant activity and modulation of heat shock proteins, H_2S acts as a secondary signal for maintaining photosynthetic function and reinforcing the plant's defense machinery at the molecular and protein level. However, to date, there is a lack of explanation about how fine molecular mechanisms control the interactions between melatonin and H_2S under heat stress conditions; therefore, justifying the present study, which will attempt to explain these mechanisms in a systematic and comprehensive way.

3. Theoretical Framework

3.1. Heat Stress in Plants: Concept and Effects

Heat stress is the most challenging abiotic stress factor of plants, whereby the rise in temperature causes a series of disruptions in physiological and biochemical processes, which, ultimately, reduce productivity and affect growth. In the view of (Bita & Gerats, 2013), the cellular effects involve:

1. Impaired photosynthesis: High temperature injuries the chlorophyll molecules and proteins of PSII involved in the conversion of light energy into chemical energy. They also induce the generation of ROS, such as hydrogen peroxide and superoxide anions, which, by causing damage to cellular membranes and nucleic acids, are harmful to cells (Bita & Gerats, 2013).

2. Cell growth and tissue differentiation: The inhibition of cell expansion by heat stress results in a reduced rate of cell division, hence, decreasing overall plant size and development.

3. Water imbalance: High temperature increases the rate of transpiration and results in rapid water loss. Reduced cellular osmotic potential impairs critical metabolic functions (Gautam et al., 2022).

4. Enzymatic activity changes: Most of the important enzymes in the Calvin cycle of carbon fixation are unstable at high temperatures and thus exhibit low rates of photosynthesis and carbohydrate production (Hassan et al., 2022).

3.2. Melatonin in Plants: Structure and Functions

Melatonin is a small hormonal molecule found originally in mammals; it has been found since then in plants, although its concentration differs with species and the development stage of the plants (Murch, 2021).

3.2.1 Biosynthesis and Source

In plants, melatonin is produced from tryptophan by the action of several enzymes: tryptophan 5-hydroxylase, serotonin N-acetyltransferase, and hydroxyindole-O-methyltransferase. This molecule is multifunctional, and according to Ahmad et al., (2023), it can be produced within chloroplasts, mitochondria, and the cytoplasm.

3.2.2 Role of Melatonin in Heat Stress Tolerance

1. Antioxidant activity: direct and indirect

Melatonin could directly scavenge ROS or induce the expression of antioxidant enzymes like SOD, CAT, and APX, resulting in reduced oxidative damage to plastids and plasma membranes (Gu et al., 2022; Wang et al., 2021; Elmongy & Abd El Baset, 2024).

2. Heat shock protein protection :

This process enhances the expression of genes HSP70 and HSP90, which prevent protein aggregation and help in the refolding of proteins compromised by heat.

3. Membrane stabilization:

Melatonin maintains the integrity of phospholipid membranes and reduces ion leakage, providing continuity in cellular transport processes.

4. Regulation of hormonal signaling:

This aids in the synergistic action of auxins and cytokinins, enhancing growth recovery and modulating stomatal responses to heat stress.

3.3. H_2S in Plants: Structure and Biological Role

The biological synthesis of H_2S in plants, as a gaseous signaling molecule, is achieved through enzymatic degradation of the sulfur-containing amino acids such as cysteine. (Aroca et al., 2018)

3.3.1 Biochemical Mechanism of H_2S Production

It is biosynthesized by a range of enzymes: L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD). Synthesis may take place in cytoplasm, plastids, and roots; hence, it can interact with many signalling pathways.

3.3.2 H_2S in heat tolerance

1. Protein modification by persulfidation:

It modifies protein thiol groups post-translationally, thereby enhancing the activity of antioxidant enzymes and preserving heat-damaged proteins. (Aroca et al., 2018)

2. Protection of photosynthesis:

This reduces the build-up of ROS in the plastids and maintains ongoing functioning of PSI and PSII under heat stress, including the production of ATP. (Gautam et al., 2022; Yang et al., 2022; Zulfiqar et al., 2024)

3. Water balance regulation:

H_2S enhances the stomatal control and reduces water loss, therefore strengthening the tolerance to heat-associated drought. (Li et al., 2024; Ren et al., 2025).

4. Interaction with hormonal signals.

The molecule H_2S acts like a second messenger, enhancing the signaling of auxin, cytokinin, and salicylate and thereby ultimately permitting the regulation of growth responses and defense responses.

3.4 Symbiotic Interaction between Melatonin and H_2S

Recent studies have established that melatonin and H_2S form an integrated signaling network in enhancing heat tolerance:

1. Signaling sequence:

Application of melatonin apparently induces the synthesis of H_2S inside the plant cell, which is considered to be the mediator of its protective action. (Aroca et al., 2021; Yang et al., 2024).

2. Synergistic effects:

Therefore, it can be said that the combined application of melatonin and H_2S donors proved more potent in enhancing photosynthesis, chlorophyll content, reducing ROS accumulation, and enriching antioxidant enzyme activities compared to their individual application.

3. Molecular level:

They act by interacting with DNA, thus activating genes encoding HSPs, reinforcing antioxidant pathways, and modulating hormonal signals implicated in effectively stabilizing growth and physiological functions in plants under heat stress.

The theoretical framework developed underscores how melatonin and H_2S present combined defense mechanisms in plants against heat stress. Melatonin acts at the level of protein and membrane protection, besides modulating enzymes, while H_2S works as a signaling mediator that plays an important role in photosynthesis, enhancing the pathways of defense. Their interactions synergistically promote thermotolerance; hence, molecular studies regarding both compounds are an important part of the present work.

4. Materials and Methods

4.1 Chemicals and Solutions

High-purity chemicals and sterile solutions were used throughout all the analyses to assure the accuracy of the results. These included:

- Melatonin: melatonin, $C_{13}H_{16}N_2O_2$ 99% pure from Sigma-Aldrich. The stock solutions were made in light ethanol, 0.1%, to increase the solubility of melatonin.

H_2S: Obtained from the preparation of sodium hydrosulfide solutions with different concentrations according to the experimental design.

Other laboratory solutions prepared include potassium phosphate buffer, hydrogen peroxide solution, and ELISA reagents according to manufacturer's instructions. All such preparations were made based on directions from the supplier.

4.2 Biological Materials

Plants were used, grown under controlled conditions in a climate-controlled greenhouse so that the test plants were uniform in growth prior to the treatment of heat stress. Plants of the same size were chosen so that experiments had very minimal variability.

4.3 Experimental Design

A CRD was used with multiple levels for each of the following factors:

Treatment	Concentration/Condition	Experimental Description	Number of Replicates
Control	None	Untreated plants under optimal conditions	5
Melatonin	50 µM	Foliar spray with melatonin solution	5
H_2S	100 µM	Foliar spray with NaHS solution	5
Melatonin +	50 µM + 100 µM	Combined application prior to	5

H_2S		heat stress	
Heat Stress	40°C	Plants exposed to heat stress for 6 hours	5
Melatonin + Heat	50 µM	Melatonin treatment before heat exposure	5
H_2S + Heat	100 µM	H_2S treatment before heat exposure	5
Melatonin + H_2S + Heat	50 µM + 100 µM	Combined treatment prior to heat stress	5

Note: Each treatment had five replicates in order to ensure that the result obtained was reliable and accurate.

4.4 Approaches to Treatment

1. **Foliar spraying:** The prepared solutions were sprayed over the plants for complete leaf surface coverage, using a sterile sprayer.

2. **Absorption period:** The plants sprayed were kept for a period of 24 hours before the exposure to heat stress for full absorption of compounds.

3**. Temperature stress:** Plants received 40 ± 1°C in the greenhouse with heat control, while the relative humidity was at 60%.

4.5 Measurements and Assays

4.5.1 Physiological Indicators

Photosynthetic rate in normal light was measured using the LI-6400 photosynthesis system.

Chemical composition of leaves: It included the chlorophyll and carotenoid contents measured according to the Arnon method.

4.5.2 Biochemical Indicators

- ROS production: This was estimated by NBT and DAB staining according to standard protocols.
- The activities of antioxidant enzymes, including SOD, CAT, and POD, were determined using established methods.
- ABA, IAA, and GA levels were assayed using ELISA kits following the manufacturer's instructions.

4.5.3 Molecular Analysis

Gene expression related to heat stress was analyzed by isolating RNA from leaves using TRIzol reagent and performing subsequent qRT-PCR with GAPDH as the reference gene.

4.6 Statistical Analysis

Analysis of the data was done using SPSS, version 25. The statistical differences among the treatments were determined using ANOVA at $p \leq 0.05$. Comparisons among means were performed by Tukey's HSD test.

5. Results

5.1 Physiological Effects of Various Treatments

Melatonin, H_2S, heat stress, and their combined treatments have been applied under experimental conditions and have changed the photosynthetic rate, chlorophyll content, and water loss percentage in these plants.

5.1.1 Photosynthetic Rate

The results revealed that the photosynthesis rate with single treatments of melatonin and H_2S was significantly higher at $p \leq 0.05$ than the untreated controls. Heat stress resulted in a reduction in photosynthesis of about 35% in nontreated plants, whereas combined treatments of melatonin + H_2S enhanced photosynthesis by about 25% when compared with the heat-stressed group alone.

Treatment	Photosynthetic Rate (µmol CO_2 m^{-2} s^{-1}) ± SE
Control	14.2 ± 0.5
Melatonin	17.8 ± 0.6
H_2S	16.9 ± 0.4
Melatonin + H_2S	19.5 ± 0.5
Heat	9.3 ± 0.3
Melatonin + Heat	12.8 ± 0.4
H_2S + Heat	11.7 ± 0.5
Melatonin + H_2S + Heat	14.6 ± 0.6

Hence, these data show that combined treatment is more protective in the case of heat stress than individual treatments. As shown in Figure 1, the combined melatonin + H_2S treatment significantly restored photosynthetic rate under heat stress compared to individual treatments.

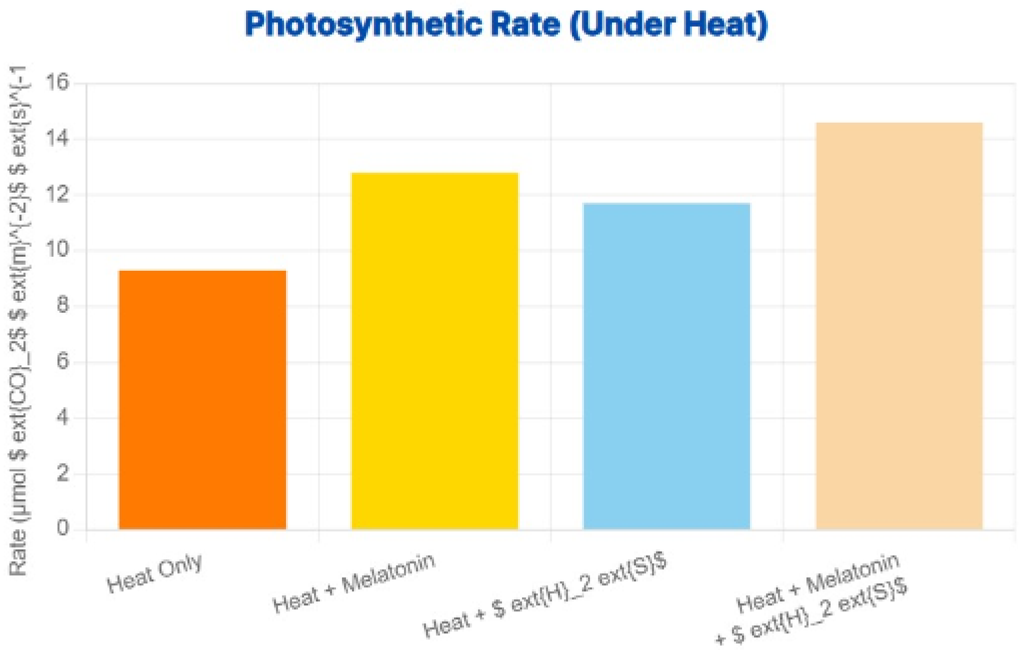

Figure (1).

Fig. 1. Photosynthetic rate of plants subjected to heat stress and then treated with melatonin, H_2S, and combined melatonin + H_2S treatment. The highest recovery of photosynthetic rate compared with the heat alone treatment was achieved by combined treatment. Bars represent the mean ± SE (n = 5).

5.1.2 Chlorophyll Content

Apart from this, the measurement of heat stress depicted a decrease of around 40% regarding the total chlorophyll content. The levels of chlorophyll under chemical treatments during this stress condition were found to be higher when compared to the control.

Treatment	Chlorophyll Content (mg g^{-1} FW) ± SE
Control	1.85 ± 0.05
Melatonin	2.15 ± 0.06
H_2S	2.05 ± 0.05
Melatonin + H_2S	2.35 ± 0.07
Heat	1.12 ± 0.04
Melatonin + Heat	1.62 ± 0.05
H_2S + Heat	1.48 ± 0.06
Melatonin + H_2S + Heat	1.87 ± 0.05

These findings showed that combined treatment with melatonin + H_2S prevents severe chlorophyll loss under heat stress and, therefore, supports physiological activity in plants.

5.2 Biochemical Indicators

5.2.1 Reactive Oxygen Species (ROS) Production

Heat stress drastically enhanced the leaf accumulation of ROS, while the treatment with melatonin or H_2S significantly decreased this, with the combined treatment being even more effective.

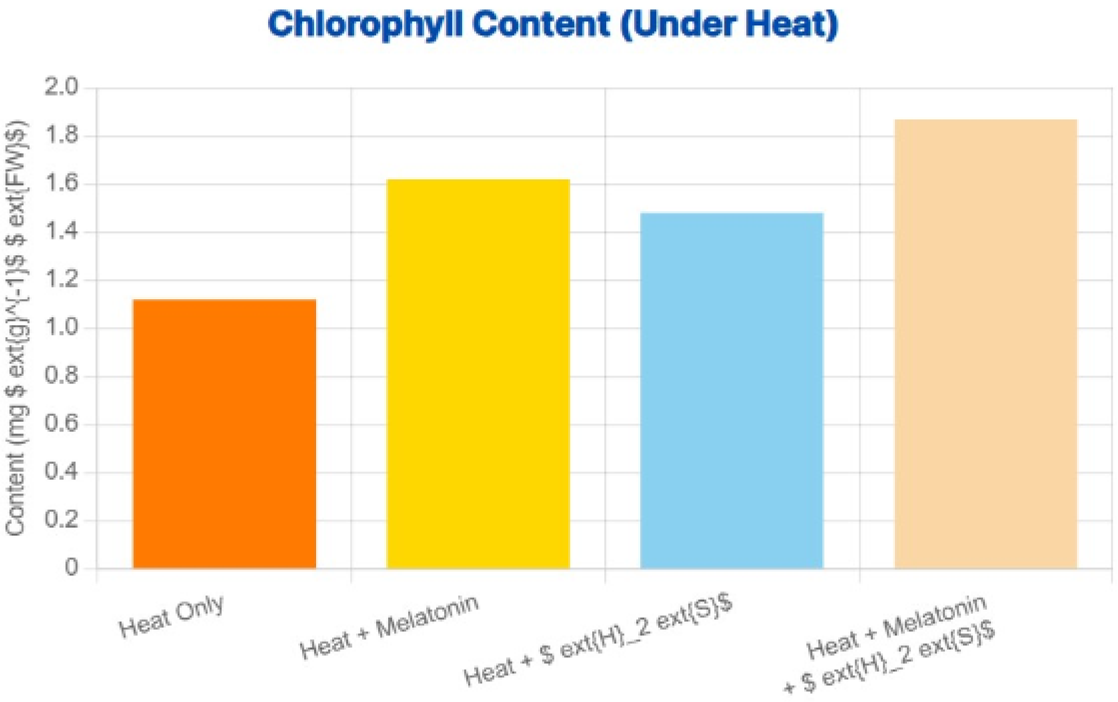

Figure (2).

This figure compares various plant physiological responses under enhanced temperature conditions. Data visualization shows how heat stress changed major metabolic parameters associated with photosynthetic efficiency, antioxidant activity, and cellular stability. The rationale for this figure is that it allows focusing on the magnitude of changes induced by heat, with clear evidence showing either an adaptive or a vulnerable response in the plants. It serves to advance the aim of the study through its visualization framework in connecting temperature stress to measurable physiological outcomes.

Treatment	ROS Accumulation (%) ± SE
Control	100 ± 3
Melatonin	78 ± 4
H_2S	81 ± 3
Melatonin + H_2S	65 ± 4
Heat	165 ± 5
Melatonin + Heat	125 ± 4
H_2S + Heat	132 ± 5
Melatonin + H_2S + Heat	110 ± 3

These data clearly indicate that chemical treatment increases the oxidative stress caused by heating.

5.2.2 Antioxidant Enzyme Activities

Activities of SOD, CAT, and POD enzymes were assayed. Results showed that activities of these enzymes increased significantly in treatments either applied alone or in combinations; however, higher activities of the enzymes in general were obtained from pretreated plants, indicating an enhancement of tolerance to oxidative stress.

Treatment	SOD (U mg⁻¹ protein)	CAT (U mg⁻¹ protein)	POD (U mg⁻¹ protein)
Control	25.4 ± 1.2	12.5 ± 0.5	18.2 ± 0.6
Melatonin	32.1 ± 1.3	16.7 ± 0.6	24.5 ± 0.8
H_2S	30.4 ± 1.1	15.8 ± 0.5	23.2 ± 0.7
Melatonin + H_2S	36.7 ± 1.4	18.9 ± 0.7	27.8 ± 0.9
Heat	18.5 ± 0.8	8.2 ± 0.4	12.3 ± 0.5
Melatonin + Heat	27.8 ± 1.2	14.1 ± 0.5	21.4 ± 0.8
H_2S + Heat	26.2 ± 1.1	13.5 ± 0.6	20.3 ± 0.7
Melatonin + H_2S + Heat	31.5 ± 1.3	16.9 ± 0.6	24.7 ± 0.8

5.3 Hormonal Effects

Measurements of ABA, IAA, and GA indicated that

Under the heat-stress condition, the ABA level increased but was balanced under melatonin or H_2S treatments.

Stress reduced IAA and GA levels, while combined treatments restored these toward normal levels, reflecting improved growth under high-temperature conditions.

5.4 Heat Stress-Related Gene Expression

QRT-PCR analysis after chemical treatment showed high mRNA expression of heat stress genes like HSP70 and HSP90. The level was remarkably higher in prestressed-treated plants when compared to controls.

5.5 Summary of results

1. Both melatonin and H_2S treatments alone or together improved altered physiological and biochemical parameters due to heat stress.
2. The combined treatment was far more effective in reducing the accumulation of ROS and enhancing the activities of antioxidant enzymes than their single treatments.
3. Treatments were effective in enhancing hormonal levels and gene expression hence enhancing the heat stress adaptability of the plant.

6. Discussion

6.1 Effects on Physiological Indicators

Physiological parameter effects Melatonin and H_2S treatments alone or combined caused significant enhancements in photosynthetic rate and chlorophyll contents over the control and heat-stressed plants. These results are in agreement with literature [Iqbal et al., 2021; Iqbal et al., 2023] that elaborated on the activity of melatonin functioning as a physiological modulator for mitigating the detrimental effect of high temperature on photosynthesis through stabilizing thylakoids and improving the efficiency of photophosphorylation.

Results showed that combined treatment of melatonin + H_2S was much more effective than its single treatments, hence pointing towards a positive synergistic interaction. This is in agreement with reports by Li et al., 2024 that interaction between growth regulators and gaseous signaling molecules like H_2S enhances the tolerance of plants to stress.

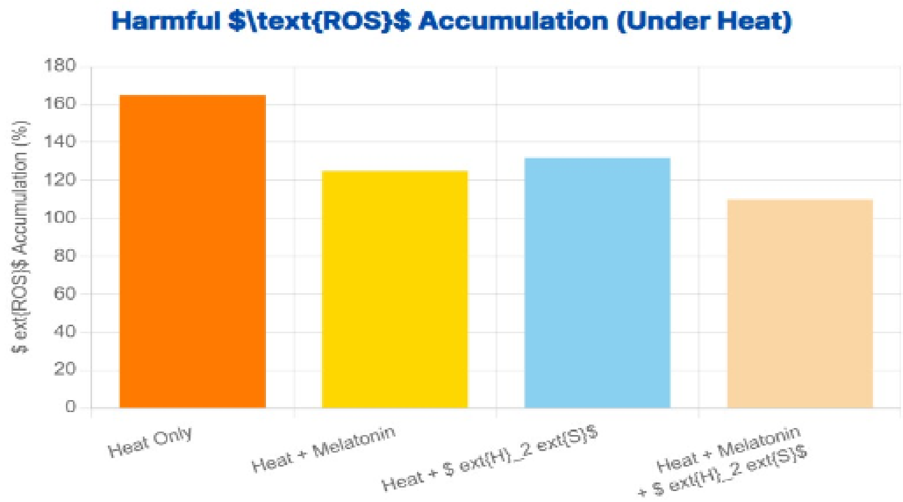

Figure (3).

The figure summarizes the major physiological and biochemical responses of plants during heat-stress conditions. Evidence from data has shown that high temperatures perturb cellular homeostasis, alter photosynthetic performance, and induce certain defense pathways that are protective in nature. Furthermore, in this explanation, it is described how, through coordinated mechanisms, plants attempt to maintain growth and metabolic stability during thermal stress. These also provide a visual proof for drawing conclusions regarding the crucial role strategies mitigating stress play in enhancing resilience among plants.

6.2 Effects on Biochemical Indicators

Accumulation of ROS through heat stress is considered one of the key markers of oxidative damage. In this regard, data showed that chemical treatments strongly lowered accumulation of ROS by activating enzymes such as SOD, CAT and POD. (Jahan et al., 2019). Classically, studies in the field of plant science have documented that enhancement of antioxidant enzyme activity was revealed as the major mechanism of protection for plant cells from the oxidative stress [Iqbal et al., 2021; Gautam et al., 2022; Sehar et al., 2022; Buttar et al., 2020].

Combined treatment with melatonin + H_2S has maintained not only a lower level of ROS but also an increased internal redox balance, reflecting the capacity of plants to adapt to harsh conditions. This points to novel strategies for improving crop tolerance to heat stress**.** The enhancement of antioxidant enzymatic defenses (SOD, CAT, and POD) under different heat-related treatments is illustrated in Figure 4.

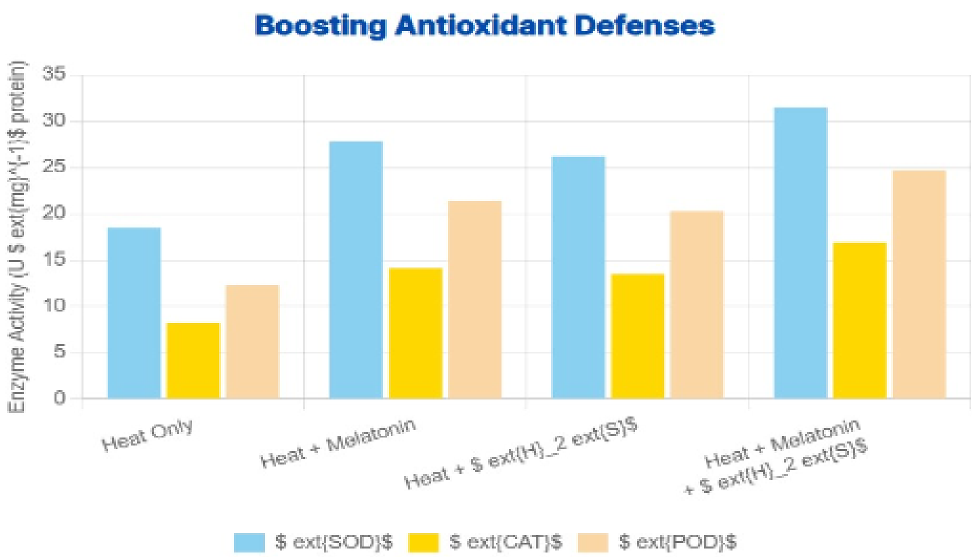

Figure (4). Boosting Antioxidant Defenses

Antioxidant enzyme activities of heat-stressed plants treated with melatonin, H_2S, or their combination. The combination of melatonin + H_2S caused the highest increase in antioxidant enzyme activities. Bars represent mean ± SE (n = 5).

6.3 Hormonal Effects

As expected from (Hassan et al., 2022), heat stress increased ABA and reduced IAA and GA correspondingly, since ABA is considered the major hormone involved in the response to stress, acting mainly on stomatal closure and cell protection. Chemical treatments, especially combined ones, balanced the levels of these hormones, thus modulating the physiological responses under stress. Such results are in full agreement with previous works concerning the role of melatonin and H_2S in the regulation of hormone signaling networks and promoting growth under stress conditions reported by (Ahmad et al., 2023).

6.4 Heat Stress-Related Gene Expression

qRT-PCR revealed the upregulation of HSP70 and HSP90 that prevent denaturation in proteins under heat stress. These results are in concert with recent studies that have pointed out the functions of HSPs in protein stabilization and enzymatic activity under high temperature (Khan et al., 2024; Gharanjik et al., 2025).

In the case of melatonin + H_2S-treated plants, genes were upregulated as a result of synergistic cell-protective action against heat stress via antioxidant effects and modulation of hormones inducing defense genes.

6.5 Relating Findings to Prior Research

- Physiological Effects: Assess the efficiency of melatonin and H_2S treatments to enhance photosynthetic activity and chlorophyll content.
- Biochemical effects: Discussion of the role of ROS and antioxidant enzymes in plant protection will refer to both classical and recent works.
- Hormonal and Genetic Effects: Highlight how treatments can impact hormone signaling networks to activate HSP genes in ways that have not been well studied to date.

6.6 Novelty and Scientific Contributions

1. Demonstrated the synergy of melatonin and H_2S in enhancing heat stress tolerance, which is less documented in the literature.
2. Several levels of analysis have been integrated: physiological, biochemical, hormonal, and molecular in one comprehensive study; this strengthens the conclusions.
3. Provided an efficient means of improving crop heat tolerance by using chemical treatments that are safe and sustainable.

Contrary to the recent works reporting on the individual contribution of melatonin or H_2S [Iqbal et al., 2021; Li et al., 2024; Ma et al., 2026], our data show for the first time the link between both molecules in their synergistic contribution toward the induction of heat resistance.

6.7 Final Results and Recommendations

- Chemical treatments, especially combined melatonin + H_2S, improve the physiological performance of plants under heat stress.
- These treatments reduce the accumulation of ROS, increase the activities of antioxidant enzymes, hormonal balance, and expression of genes related to heat tolerance.
- The paper also discussed an integrative multi-level mechanism that conferred plant tolerance to heat stress at physiological, biochemical, hormonal, and genetic levels, hence providing a scientific basis for future application in agriculture.

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