IMPLICATIONS OF HYDROGEN SULFIDE DURING SODIUM CHLORIDE STRESS IN SOME VEGETABLE CROPS

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DECLARATION

I, hereby declared that the presented work in the thesis entitled "Implications of hydrogen sulfide during sodium chloride stress in some vegetable crops" in fulfilment of degree of Doctor of Philosophy (Ph. D.) is outcome of research work carried out by me under the supervision of Dr. Vineet Kumar, working as Professor, in the Department of Biotechnology, School of Bioengineering and Biosciences of Lovely Professional University, Punjab, India. In keeping with the general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigators. This work has not been submitted in part or in full to any other University or Institute for the award of any degree.

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CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled "Implications of hydrogen sulfide during sodium chloride stress in some vegetable crops" submitted in fulfillment of the requirement for the award of degree of Doctor of Philosophy (Ph.D.) in the Department of Botany, School of Bioengineering and Biosciences, is a research work carried out by Assima Firdoos, 12009918, is bonafide record of his/her original work carried out under my supervision and that no part of thesis has been submitted for any other degree, diploma or equivalent course.

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ABSTRACT

Soil salinity has emerged as a major threat to global food security, posing critical challenges to agricultural sustainability. By 2050, climate change and anthropogenic activities are projected to render half of India's arable land salt-affected, with documented evidence showing a 41% reduction in vegetable production from 1965 to 2016, a trend accelerating as approximately 10% of additional agricultural land is getting salinized annually. The primary mechanism of crop productivity reduction is osmotic stress, whereby elevated salt ion concentrations increase soil osmotic potential, inhibiting water absorption and triggering metabolic dysfunctions, including membrane degradation, reactive oxygen species accumulation, ionic imbalance, and decreased photosynthetic capacity. Plants have evolved adaptive strategies such as osmotic homeostasis maintenance, ionic transport regulation, and antioxidant machinery upregulation, these endogenous defense mechanisms prove insufficient against severe stress conditions, necessitating the development of innovative, sustainable agricultural interventions to address projected food production demands for a population anticipated to reach 9.7 billion by 2050, demanding a 25-70% rise in global food production through enhanced understanding of plant stress responses and effective sustainable remediation strategies. The application of exogenous substances that can strengthen the plant's defense mechanism represents an effective strategy to lessen the detrimental impact of salt stress on plants. Hydrogen sulfide (H_2S) is one such regulator, documented as a potent gaseous signaling molecule that plays a major regulatory role in improving the resistance of plants against stress. Furthermore, it offers an affordable and environmentally safe approach.

The vegetable sponge gourd (*Luffa aegyptiaca* Mill.) and okra (*Abelmoschus esculentus* L.) are summer vegetable crops with great economic value. These vegetables are farmed for their fruit across the globe, contributing significantly to global food security. Both of these vegetables are vulnerable to salinity, therefore making it crucial to gain insights into their salt stress mitigation strategies for effective crop management to boost productivity.

The present study examined the possible underlying ameliorative mechanism of H_2S on physiological and biochemical parameters of sponge gourd and okra under sodium chloride (NaCl) stress conditions. Further to validate the role of H_2S emitted from

sodium hydrogen sulfide (NaHS) as an abiotic stress-tolerant molecule under NaCl stress, its scavenger, hypotaurine (HT), and inhibitor DL-propargylglycine (PAG) were also used in this study. Few studies have documented the role of H_2S in stress regulation, but how H_2S affects NaCl stress regulation in sponge gourd and okra still requires investigation. This necessitates looking into the regulatory mechanisms that support the functions of H_2S as a NaCl stress-mitigating signaling molecule in sponge gourd and okra. Hence, we hypothesize the role of H_2S in regulating the NaCl stress-mediated oxidative damage and nitrogen metabolism in sponge gourd and okra seedlings for NaCl stress mitigation.

NaCl stress was observed to negatively influence several growth parameters, including fresh weight, shoot length, and root length of sponge gourd and okra seedlings. Reduced photosynthetic pigments, chlorophyll a, carotenoids, and chlorophyll b, were observed to impair carbohydrate and protein content in both sponge and okra seedlings exposed to NaCl stress. NaCl stress triggered the accumulation of oxidative stress markers, malondialdehyde and hydrogen peroxide. Further NaCl stress elicited notable stress-adaptive responses in both sponge gourd and okra seedlings, characterized by the accumulation of non-enzymatic (phenol, and flavonoids) and enhanced activity of enzymatic antioxidants (superoxide dismutase, ascorbate peroxidase, and catalase).

NaCl stress significantly impaired nitrogen metabolism, as evidenced by reduced nitrate and nitrite content, alongside diminished activity of nitrate assimilation enzymes (nitrate and nitrite reductase) and ammonia assimilation enzymes (glutamate synthetase and glutamine synthetase) in both plants. However, ammonia accumulation increased under NaCl stress, which corresponds with enhanced activity of glutamate dehydrogenase, suggesting an alternative metabolic pathway activated under NaCl conditions in sponge gourd and okra.

Exogenous supplementation of H_2S was observed to reduce salinity-induced stress in sponge gourd and okra by increasing endogenous H_2S production, thereby enhancing antioxidative enzyme activities, lowering reactive oxygen species (ROS) buildup, and boosting growth traits. The H_2S supplementation also improved photosynthetic efficiency and enhanced the synthesis of carbohydrates, proteins, and non-enzymatic antioxidants in both plants. Furthermore, H_2S exposure to NaCl-stressed okra and sponge gourd seedlings improved inorganic nitrogen content and upregulated nitrogen

and ammonia assimilating enzymes, including nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthetase. The application of HT and PAG halts the production and accumulation of endogenous H_2S, thereby reversing the beneficial effect of H_2S induced on salt-stressed okra and sponge gourd seedlings.

In conclusion, the H_2S application to NaCl-stressed sponge gourd and okra seedlings triggers a remarkable constellation of adaptive responses in morphological, physiological, and biochemical parameters that effectively orchestrate a comprehensive defense system against the adverse impacts of NaCl stress. This study reveals a promising pathway in agricultural biotechnology, potentially revolutionizing cultivation practices in salt-affected regions through a sophisticated biochemical intervention that may convert susceptible crops into resilient producers, thereby addressing critical food security challenges in increasingly salinized agricultural environments worldwide.

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TABLE OF CONTENTS

Contents	Page No.
Declaration	i
Certificate	ii
Abstract	iii-v
Acknowledgement	vi-vii
Table of contents	viii-xiv
List of tables	xv
List of figures	xvi-xix
List of appendices	xx-xxi
Chapter 1	
1. INTRODUCTION	2-7
Chapter 2	
2. REVIEW OF LITERATURE	9
2.1. The current state of soil salinity around the world	9-10
2.2. Causes of salinity	10-11
2.3. Categorization of salinity	12
2.4. Effect of salt on growth attributes	13

2.4.1. Effect on germination and growth	13-16
2.4.2. Salinity effect on pigment content	17-20
2.4.3. Salinity-induced oxidative stress and its impact on the antioxidant system	20-26
2.4.4. Salinity and nitrogen metabolism	26-29
2.5. H_2S as a stress modulator	29-37
2.6. Endogenous H_2S dynamics in plants: Basal level and stress responses	37
2.7. Atmospheric H_2S and the need for exogenous H_2S donors	38
2.8. Donors of H_2S	39
2.9. Mechanism of H_2S release from NaHS	39-40
2.10. Rationale for investigating H_2S potential in NaCl stress mitigation (Hypothesis)	40-41
2.11. Objectives	41
Chapter 3	
3. METHODS AND MATERIALS	43
3.1. Test crops	43
3.2. Growth conditions of sponge gourd and okra seedlings	43
3.3. Chemicals, glassware, and instruments	43-44
3.4. Nutrient media	44-45

3.5. Sterilization process of growing media and glassware	45
3.6. Preparation and mode of NaCl, NaHS, PAG, and HT treatment	45-46
3.7. Treatment design of experiment	46
3.8. Evaluation of morphological and biochemical attributes	47
3.8.1. Estimation of morphological characteristics	47
3.8.2. Quantification of photosynthetic pigments	47
3.8.3. Assessment of carbohydrate content	48
3.8.4. Assessment of protein content	48-49
3.8.5. Determination of nitrogen metabolism	49
3.8.5.1. Estimation of inorganic nitrogen content	49
3.8.5.1.1. Determination of nitrate content	49
3.8.5.1.2. Determination of nitrite content	49-50
3.8.5.1.3. Determination of ammonia content	50-51
3.8.5.2. Nitrate assimilation enzymes	51
3.8.5.2.1. Quantification of nitrate reductase activity	51-52
3.8.5.2.2. Quantification of nitrite reductase activity	52
3.8.5.3. Ammonia assimilation enzymes (GS-GOGAT pathway)	52

3.8.5.3.1. Determination of the activity of glutamine synthetase	52-53
3.8.5.3.2. Determination of the activity of glutamate synthase	54
3.8.5.3.3. Determination of glutamate dehydrogenase activity	54-55
3.8.6. Measurement of oxidative stress	55
3.8.6.1. Estimation of hydrogen peroxide content	55-56
3.8.6.2. Estimation of lipid peroxidation via Malondialdehyde content	56
3.8.7. Assessment of enzymatic and non-enzymatic antioxidants	57
3.8.7.1. Enzymatic antioxidants	57
3.8.7.1.1. Determination of superoxide dismutase activity	57
3.8.7.1.2. Determination of ascorbate peroxidase activity	57-58
3.8.7.1.3. Determination of catalase activity	58
3.8.7.2. Nonenzymatic antioxidants	59
3.8.7.2.1. Assessment of total phenolic content	59
3.8.7.2.2. Assessment of flavonoid content	59-60
3.8.8. Assessment of antioxidant potential (DPPH assay)	60
3.8.9. Determination of H_2S content	60-61
3.8.10. Statistical analysis	61

Chapter 4	
4. RESULTS AND DISCUSSION	63
4.1. Determination of appropriate salt concentrations to be used for the sponge gourd study	63-65
4.2. Determination of NaHS concentration for the sponge gourd study	66-68
4.3. Morphological characterization of sponge gourd exposed to mixed treatments in hydroponics	68-71
4.4. NaHS-mediated rescue of photosynthetic pigments, biomass, and carbohydrate accumulation	71- 74
4.5. NaCl and NaHS mediated altered accumulation of total polyphenols, flavonoids, and antioxidant potential	74-76
4.6. Altered accumulation of enzymatic antioxidant parameters in sponge gourd	76-79
4.7. NaHS rescued the hydrogen peroxide and lipid peroxidation content of salt-stressed sponge gourd	79-80
4.8. Altered protein content in variously treated sponge gourd	81-82
4.9. Effect of varied treatment on nitrogen metabolism of sponge gourd	82
4.9.1. Enhanced inorganic nitrogen content on NaHS supplementation of salt-stressed sponge gourd	82-84
4.9.2. NaHS altered enzymes involved in nitrogen metabolism: nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, and glutamate dehydrogenase.	85-88
4.10. H_2S content augmented on NaHS exposure	89-91

4.11. Determination of salt concentrations to be used for the okra study	92-94
4.12. Determination of NaHS concentrations for the okra study	94-97
4.13. Evaluation of okra morphology subjected to a combination treatment in hydroponics	97-100
4.14. NaHS-mediated rescue of photosynthetic pigments, biomass, and carbohydrate content of NaCl-stressed okra	100-103
4.15. Polyphenol, flavonoid, and antioxidant responses of okra seedlings to NaCl and NaHS treatment	103-105
4.16. Enzymatic antioxidant responses of Okra	105-107
4.17. Oxidative stress parameters were regulated in NaCl-stressed okra on NaHS treatment	108-110
4.18. Altered protein content in okra	110-111
4.19. Regulation of nitrogen metabolism by NaHS in NaCl-stressed okra seedlings	111
4.19.1. Analysis of inorganic nitrogen content in salt-stressed okra seedlings on varied exposure	112-114
4.19.2. Analysis of nitrogen metabolism enzymes in salt-stressed okra seedlings on varied exposure.	114-119
4.20. Analysis of H_2S content in okra subjected to varied treatments	119-121
Chapter 5	
5. SUMMARY AND CONCLUSION	123-127
BIBLIOGRAPHY	128-169

DETAILS OF PUBLICATION	170
CONFERENCES	171-172
WORKSHOPS	173
ANNEXURE	174-175

LIST OF TABLES

Table No.	Title	Page No.
2.1	Effects of NaCl stress on plant growth attributes.	15-16
2.2.	The impact of NaCl stress on pigment, protein, and carbohydrate content in plants.	18-20
2.3.	Represents the impact of NaCl stress on oxidative homeostasis and the antioxidant system.	23-26
2.4.	Impact of NaCl toxicity on inorganic nitrogen content and nitrogen metabolism enzymes.	28-29
2.5.	Represents the salt stress mitigating potential of H_2S in plants.	33-37
3.1.	Represents the nutritional media composition.	44-45
4.1.	Influence of varying NaCl concentrations on sponge gourd.	65
4.2.	Influence of NaHS exposure on sponge gourd.	67
4.3.	Experimental setup used in the present study to determine the potential of NaHS as an H_2S donor for inducing salt stress tolerance in sponge gourd.	68
4.4.	Effects of different NaCl concentrations on okra. Elevated NaCl concentrations drastically diminished the morphological and biochemical parameters under investigation.	94
4.5.	Effect of NaHS exposure on okra. N2 (100 µM) improved the measured morphological and biochemical plant characteristics.	96-97
4.6.	Experimental setup for investigation of NaHS efficacy as an H_2S donor to induce salt stress endurance in okra seedlings.	97

LIST OF FIGURES

Figure No.	Content	Page No.
2.1.	Factors affecting soil salinity.	11
2.2.	Depicts the detrimental consequences of salt stress on plants.	14
2.3.	An outline of endogenous H_2S synthesis in plants.	31
3.1.	The figure provides a schematic representation of the experimental layout employed in the present study.	46
4.1.	The figure depicts the effect of NaCl on the morphological parameters of sponge gourd seedlings.	64
4.2.	The figure depicts the potential of NaHS in improving sponge gourd morphology.	66
4.3.	(A) The figure depicts the potential of NaHS for maintaining the morphological parameters of salt-stressed sponge gourd. The graphs show the significant rescue of (B) shoot length and (C) root length of salt-stressed sponge gourd on NaHS supplementation.	69
4.4.	Influence of NaCl, NaHS, HT, and PAG on the photosynthetic parameters of sponge gourd. The bar graphs show the considerable enhancement in the levels of (A) chlorophyll a, (B) chlorophyll b, (C) carotenoids, (D) fresh biomass, and (E) carbohydrates in salt-stressed sponge gourd on NaHS exposure.	74
4.5.	The graphs depict the significant alterations in the (A) total phenols and (B) flavonoid accumulation of NaHS-exposed salt-stressed sponge gourd, which lead to (C) an enhanced antioxidant potential on NaHS exposure.	76
4.6.	Influence of NaCl, NaHS, HT, and PAG on the activities of (A) superoxide dismutase, (B) ascorbate peroxidase, and (C) catalase. NaHS was found to enhance the activities of superoxide dismutase, catalase, and ascorbate peroxidase, thus inducing enhanced salt stress tolerance in sponge gourd.	77

4.7.	The bar graphs depict the influence of NaHS supplementation on the oxidative stress markers (A) hydrogen peroxide, and (B) malondialdehyde, representing lipid peroxidation. NaHS exposure was found to significantly regulate the levels of both hydrogen peroxide and malondialdehyde, thus indicating its potential role in salt stress tolerance of sponge gourd.	79
4.8.	The figure shows the effect of NaHS supplementation on the protein accumulation of salt-stressed sponge gourd. The NaCl exposure has reduced the protein content of sponge gourd, but the simultaneous NaHS exposure considerably increased the content. Further, exposure of scavenger HT and inhibitor PAG in addition to NaHS suggested the potential role of H_2S for the observed variation in protein accumulation of sponge gourd.	81
4.9.	Effect of NaCl, NaHS, HT, and PAG on the metabolites of nitrogen metabolism. The bar graphs show the considerable enhancement in the accumulation of (A) nitrate and (B) nitrite in salt-stressed sponge gourd on NaHS exposure. However, NaHS exposure was found to reduce the (C) ammonia levels of salt-stressed sponge gourd, indicating stress tolerance.	83
4.10.	Effect of NaCl, NaHS, HT, and PAG on the enzymes of nitrogen metabolism. The graphs represent NaHS-mediated enhanced activity of (A) nitrate reductase, (B)nitrite reductase, (C) glutamine synthetase, and (D) glutamate synthase in salt-stressed sponge gourd, indicating NaHS-mediated rescue of nitrogen metabolism under stressed conditions. On the contrary, the activity of (E) glutamate dehydrogenase was considerably reduced on NaHS exposure, indicating its potential role in stress regulation of sponge gourd.	86
4.11.	NaHS is considered a probable H_2S donor. The bar graph shows the enhanced accumulation of endogenous H_2S in salt-stressed sponge gourd on NaHS exposure. So, hydroponic exogenous exposure of NaHS was a successful mode of ameliorating H_2S levels in sponge gourd to induce salt stress tolerance.	90
4.12.	The figure illustrates the impact of NaCl on the morphological attributes of okra seedlings.	92
4.13.	The figure illustrates the potential of NaHS for maintaining the morphological parameters of the okra.	95

4.14.	(A)The figure illustrates the efficacy of NaHS in preserving the morphological characteristics of okra subjected to salt stress. The graphs illustrate the significant improvement in (B) shoot length and (C) root length of salt-stressed okra on NaHS supplementation in the absence of H_2S scavenger and inhibitor.	99
4.15.	Impact of NaCl, NaHS, HT, and PAG on the photosynthetic attributes of okra. The bar graphs illustrate the significant improvement in the levels of (A) chlorophyll a, (B) chlorophyll b, (C) carotenoids, (D) fresh biomass, and (E) carbohydrates in salt-stressed okra on NaHS supplementation.	103
4.16.	Graphs illustrate considerable changes in the (A) total phenols and (B) flavonoid accumulation in NaHS, salt, HT, and PAG-treated okra, resulting in an (C) increased antioxidant potential due to NaHS supplementation.	105
4.17.	Impact of NaCl, NaHS, HT, and PAG on the activities of (A) superoxide dismutase, (B) ascorbate peroxidase, and (C) catalase. NaHS was observed to augment the activities of superoxide dismutase, catalase, and ascorbate peroxidase, hence promoting increased resilience to salt stress in okra.	106
4.18.	The bar graphs illustrate the effect of NaHS supplementation on oxidative stress markers (A) Hydrogen peroxide, and (B) Malondialdehyde, indicative of lipid peroxidation. NaHS exposure considerably modulated the amount of hydrogen peroxide and malondialdehyde, indicating its potential involvement in enhancing salt stress tolerance in okra.	109
4.19.	The illustration depicts the impact of NaHS exposure on protein accumulation in salt-stressed okra. NaCl exposure decreased the protein content of okra, whereas concurrent NaHS exposure significantly enhanced it. The simultaneous exposure of scavenger HT, inhibitor PAG, and NaHS indicated a probable function of H_2S in the observed alterations of protein build-up in okra.	111
4.20.	Impact of NaCl, NaHS, HT, and PAG on the inorganic nitrogen content. The bar graphs illustrate the significant increase in the levels of (A) nitrate and (B) nitrite in salt-stressed okra following exposure to NaHS. NaHS supplementation was observed to decrease the (C) ammonia levels of salt-stressed okra, suggesting enhanced stress tolerance.	113
4.21.	Impact of NaCl, NaHS, HT, and PAG on nitrogen metabolism enzymes. The graphs illustrate the NaHS-mediated enhancement of	116

	activity in (A) nitrate reductase, (B) nitrite reductase, (C) glutamine synthetase, and (D) glutamate synthase in salt-stressed okra, signifying the rescue of nitrogen metabolism under stress circumstances by NaHS. The activity of (E) glutamate dehydrogenase was significantly diminished upon exposure to NaHS, suggesting its possible involvement in the stress control of okra.	
4.22.	NaHS is regarded as a potential H_2S donor. The bar graph illustrates the increased buildup of endogenous H_2S in salt-stressed okra following NaHS exposure. The hydroponic exogenous application of NaHS effectively elevated H_2S levels in okra, thereby enhancing salt stress tolerance.	120
5.	Represents the regulation of different parameters by H_2S under salt stress.	124

LIST OF APPENDICES

Abbreviation	Description
H_2S	Hydrogen Sulfide
NaCl	Sodium Chloride
NaHS	Sodium Hydrogen Sulfide
HT	Hypotaurine
PAG	DL-Propargylglycine
EC	Electrical Conductivity
SAR	Sodium Adsorption Ratio
ESP	Exchangeable Sodium Percentage
$NaHCO_3$	Sodium Bicarbonate
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
GS-GOGAT	Glutamine Synthetase-Glutamate Synthase
DES1	L-Cysteine Desulfhydrase
DCD	D-Cysteine-Desulfhydrase
OAS	O-Acetylserine (thiol) Lyase
DSF	L-Cysteinedesulfurase
SiR	Sulfite-Reductase
CAS-C1	β-Cyanoalanine Synthase
APS	Adenosine Phosphosulfate
PLP	Pyridoxal phosphate
Na^+/H^+-antiporter	Sodium-Hydrogen Antiporter
GYY4137	Morpholin-4-ium4-Methoxy-Phenyl-(morpholino)-Phosphinodithioate
HS^-	Hydrogen Sulfide ions
S^{2-}	Sulfide ion

Na^+	Sodium ion
H^+	Hydrogen ion
KH_2PO_4	Potassium dihydrogen sulfate
$MgSO_4.7H_2O$	Magnesium sulfate
KNO_3	Potassium nitrate
$Ca\ (NO_3)_2$	Calcium nitrate
Na_2MoO_4	Sodium molybdate
$CuSO_4.5H_2O$	Copper sulfate
$ZnSO_4.7H_2O$	Zinc sulfate
$MnCl_2.4H_2O$	Manganese chloride
H_3BO_3	Boric acid
$FeCl_3$	Ferric chloride
EDTA	Ethylenediaminetetraacetic acid
HCl	Hydrochloric Acid
FW	Fresh weight
TCA	Trichloroacetic Acid
β-ME	β-Mercaptoethanol
K-P buffer	Potassium Phosphate Buffer
NEDD	N-1-Naphthylethylene diamine dihydrochloride
NADH	Nicotinamide adenine dinucleotide
ATP	Adenosine triphosphate
Na-P buffer	Sodium Phosphate Buffer
KCl	Potassium Chloride
TBA	2-thiobarbituric acid
$KMnO_4$	Potassium Permanganate
DTNB	5,5’-dithiobis-(2-nitrobenzoic acid)

Chapter 1

1. INTRODUCTION

Agriculture is the backbone of the Indian economy, employing 50% of the labor force and contributing 20.2% to the nation's GDP (Sunder, 2018; Kapil, 2021). On the global scale, agriculture engages around 39.2% (1.3 billion people) of the workforce (FAO, 2024). The agricultural revolution has introduced high-yielding crop varieties, modern irrigation techniques, and fertilizers; yet, numerous obstacles persist in Indian agrarian development. These hurdles include fragmented landholdings, suboptimal productivity, outdated farming methods, inadequate irrigation facilities, improper use of fertilizers and pesticides, and the effects of climate change. In addition, the progress of the agricultural sector has been substantially hampered by abiotic stresses, such as salinity, temperature extremes, and drought, which have diminished agricultural output by more than 50% (Foyer et al., 2016; Calanca, 2017; Cohen and Leach, 2019). About 35% of crop loss results from biotic stresses, including fungi, bacteria, viruses, and insect infestations (Savary et al., 2012). These combined pressures have led to agricultural stagnation in India, directly impacting the livelihood of farmers across the nation.

The agricultural challenges faced by India exist within a broader global context of population growth and food security concerns. As the global population is expected to reach 9.7 billion by 2050 (Cristofano et al., 2021), global food production must increase by 25-70% beyond current levels to meet anticipated demands (Hunter et al., 2017). This demographic pressure intensifies the urgency for India to address its agricultural limitations through innovative, sustainable, and climate-resilient farming practices that simultaneously support rural economies and contribute to global food security concerns.

Salinity stress is a significant environmental constraint for global agricultural productivity and is identified as the second most detrimental factor affecting vegetable crop yield (Hong et al., 2021; Behera et al., 2022). Approximately 10% of the total land area, 20% of arable land, and 50% of irrigated agricultural land are impacted by salinity worldwide (Abiala et al., 2018). It also has a significant economic impact, with annual production losses estimated at 12 billion US dollars (Zahedi et al., 2019). Multiple factors contribute to soil salinity, including climate fluctuations, low-quality irrigation, excessive groundwater extraction, and intensive farming practices (Behera et al., 2022).

Salt stress disrupts plant-environment interactions by challenging physiological homeostasis (Zhu, 2016). The accumulation of sodium and chloride ions within plant cells disturbs the crucial sodium-potassium (Na^+/K^+) balance, triggering complex physiological and biochemical responses (Zhao et al., 2021; Behera et al., 2022; Laxman et al., 2024). Biochemical changes spread across various systems, resulting in comprehensive stress reactions and compromised plant growth. Oxidative stress occurs, accompanied by inhibition of protein synthesis, enzymatic dysfunction, lipid peroxidation, and accelerated chlorophyll degradation (Machado and Serralheiro, 2017; Liang et al., 2018). Photosynthetic capacity diminishes considerably, with decreased stomatal conductance and photosynthetic rates. Furthermore, salt stress induces nutrient deficiencies by inhibiting the uptake of essential elements such as potassium, calcium, manganese, and nitrate due to interactions between salt ions and these vital mineral nutrients (Behera et al., 2022). Concurrently, under salt stress, inorganic nitrogen content and enzymes of nitrogen and ammonia assimilation were inhibited, resulting in ammonia accumulation and subsequent damaging effects on plants (Gangwar et al., 2011; Parihar et al., 2021; Raju and Prasad, 2023).

Vegetables are a rich source of nutrients and an essential component of a balanced diet (Laxman et al., 2024). However, only 30-40 of the 200 species are commonly cultivated, highlighting a gap in agricultural diversity. Globally, India ranks as the second-largest vegetable producer, following China, accounting for around 197.23 million tons of vegetables, but a 41% reduction in vegetable production has been documented from 1965 to 2016 (Scheelbeek et al., 2018; Laxman et al., 2024). By 2050, climate change and human activities are projected to cause salinity in half of India's arable land, and the annual salinisation of an additional 10% of agricultural land is expected to accelerate this alarming trend (Kumar and Sharma, 2020; Zhao et al., 2020; Altaf et al., 2023; Laxman et al., 2024). The population of India is expected to reach 1.8 billion by 2050, increasing its food grain demand to 350 million tons, necessitating the development of salt-resistant vegetable cultivars and adaptive agricultural practices to maintain productivity in increasingly salinised environments. In India, Gujrat is the state with the maximum salt affected area of 2.23 million hectare. The major driving force of soil salinisation in India is the use of brackish underground water for irrigation. *Oryza sativa* L., *Hordeum vulgare*, *Sorghum bicolor* L., *Setaria italica* L., and

Gossypium spp. are the major cultivated crops in saline and sodic soil of India. In addition to these vegetable crops, the growth and production of several other crops are also severely hampered by salinity.

The vegetable *L. aegyptiaca* Mill, sponge gourd belongs to the Cucurbitaceae family. It is a summer vegetable crop with great economic value that is farmed for its fruit across the globe. It is rich in bioactive compounds, including flavonoids, glycosides, saponins, alkaloids, phenols, sterols, triterpenes, and vitamins (Irshad et al., 2010; Partap et al., 2012; Azeez et al., 2013). These bioactive metabolites contribute to its economic, therapeutic, cosmetic, and nutritional benefits (Du et al. 2006; Partap et al., 2012; Manikandaselvi et al., 2016; Kapoor and Hasanuzzaman, 2020). In India, sponge gourd is primarily produced on low-lying hills and plains (Chandra, 1995), which makes it more prone to salt stress (Patel et al., 2017). Sponge gourd is keenly investigated because of its susceptibility to salt stress, highlighting the importance of understanding the mechanism of salt tolerance in this crop (Kapoor and Hasanuzzaman, 2020).

Another vegetable crop, *A. esculentus* L., okra, is an annual herb and a key vegetable crop of the Mallow family (Sami et al., 2013). Okra is farmed for its immature pods and contributes significantly to global food security (Andras et al., 2005; Dilruba et al., 2009). It is cultivated in tropical and subtropical regions and provides a variety of minerals, vitamins, fibers, lipids, and carbohydrates (Emuh et al., 2006; dos Santos Farias et al., 2019). Despite its high demand and nutritional value, its yield per hectare is quite poor, primarily due to soil salinity (Habib et al., 2016; Ayub et al., 2018; Naqve et al., 2021). Okra plants are particularly more vulnerable to salinity during their early phases of growth (Cerda et al., 1995).

Salinity-induced oxidative stress fundamentally affects vegetable quality and yield through cascading cellular disruptions (Kashyap et al., 2021). The cellular disturbances disrupt vital physiological processes such as water efficiency, germination, transpiration, photosynthesis, respiration, hormonal regulation, membrane permeability, antioxidant production, and nutrient dynamics (Chourasia et al., 2021; Behera et al., 2022). Despite extensive research, there are still unfilled knowledge gaps on vegetable responses to salinity (Khalid et al., 2023). Understanding salt-stress mechanisms is crucial for developing resilient vegetable varieties in increasingly

salinized agricultural environments. Besides, climate-smart measures and modified cultural practices are needed to mitigate environmental impact and achieve desired crop yields (Pathak et al., 2018; Giordano et al., 2021; Laxman et al., 2024).

An effective way to lessen the detrimental effects of salinity stress in plants is the supplementation of seeds/plants with exogenous substances, like phytohormones and signaling molecules (Dawood et al., 2022a). H_2S among such regulators is identified as a potent gaseous signaling molecule that has a key regulatory role in improving plants' endurance to stress, and it can be synthesized endogenously in plants (Jiang et al., 2019; Qi et al., 2019; Valivand et al., 2019a; Valivand and Amooaghaie, 2021a; Gautam et al., 2022; Srivastava et al., 2022). Over the last decade, there has been increased interest in H_2S due to its newly discovered roles in regulating plant growth and development across many species, including *Arabidopsis thaliana*, *O. sativa* L., *Cucumis sativus* L., *Triticum aestivum* L., *S. bicolor* L., *Zea mays*, *Glycine max*, *Spinacia oleracea*, *Musa nana*, *Malus pumila*, *Actinidia chinensis*, *S. italica* L., and many more (Parveen et al., 2017; Kaya et al., 2018; Liu et al., 2019a; Zhou et al., 2020; Hu et al., 2020; Liu et al., 2020a; Zhang et al., 2021; Wang et al., 2022a; Sun et al., 2022; Mfarrej et al., 2022; Wang et al., 2023). In contrast to high H_2S levels, poisonous to plants, suboptimal H_2S concentrations are crucial for plant development, growth, and resilience to stress (Jin et al., 2013; Fu et al., 2018).

Elevated level of H_2S has been documented in numerous plant species under stress (Younis and Mansour, 2023; Kaya et al., 2024). Research suggests that elevated H_2S can shield plants from environmental stresses such heat, cold, salt, drought, and heavy metals (Jin et al., 2013; Liu et al., 2021a; Zhang et al., 2021). H_2S was observed to trigger stomatal closure under drought stress in *Brassica rapa* L., and *A. thaliana* (Jin et al., 2017; Zhang et al., 2023a; Wang et al., 2024; Liu et al., 2021a). In response to cold stress, H_2S triggers antioxidant response and promotes the buildup of metabolic compounds, including total soluble sugar, proline, flavonoids, sucrose, and chlorophyll (Cui et al., 2025). Salt-stressed plant species, including *A. thaliana*, *Coriandrum sativum*, *Brassica oleracea, C. sativus* L., *S. italica* L., and *Capsicum annuum*, showed improved growth on H_2S exposure (Yasterb et al., 2020; Kapoor et al., 2023; Shalaby et al., 2023; Luo et al., 2023; Zhang et al., 2023b; Kaya et al., 2024). Several plant species, including *A. thaliana*, *Solanum lycopersicum* L., *O. sativa* L., *T. aestivum*,

Fragaria ananassa L., *Malus hupehensis*, *Populus popularis*, *Spartina alterniflora*, *Medicago sativa*, *Populus euphratica*, and *Avicennia marina* have had their sodium accumulation, Na^+/K^+ ratio, and potassium exocytosis decreased when exposed to high salinity conditions in the presence of H_2S (Lai et al., 2014; Mostofa et al. 2015a; Guo et al., 2018; Zhao et al. 2018; Wei et al. 2019; Ding et al., 2019; Li et al., 2020a,b ;Wei et al., 2022). Moreover, H_2S reportedly enhanced heavy metal endurance in plants by heavy metal ions fixation through upregulated synthesis of phytochelatins and metallothionein (Yu et al., 2018; Fang et al. 2014, 2016; Jia et al. 2016; Liu et al. 2016; Valivand et al., 2019b), and their transport into vacuoles (Chen et al. 2013; Wang et al. 2019; Zhu et al. 2018).
H_2S induce salt stress tolerance through a network of multiple coordinated mechanisms, such as regulating ion homeostasis (Wei et al., 2022), counteracting oxidative stress by upregulating antioxidant system (Verma et al., 2023a), modulating nitrogen metabolism (Raju and Prasad, 2023), and maintaining the synthesis and accumulation of carbohydrates, proteins, and nitrogen (Jiang et al., 2019; Rizwan et al., 2019; Jiang et al., 2020; Wei et al., 2021a; Valivand and Amooaghaie, 2021b; Liu et al., 2022a). Also, H_2S may have a beneficial effect on adventitious roots, postharvest senescence, and seed germination when subjected to abiotic stress (Chen et al., 2011; Wang et al., 2022b). These robust protective pathways position H_2S exposure as an effective and sustainable approach for improving crop performance under salinity stress conditions.

Studies have documented the role of H_2S in stress regulation; however, the economically significant crops *L. aegyptiaca* Mill. and *A. esculentus* from the family Cucurbitaceae and Malvaceae, respectively, have received less attention regarding the impact of H_2S on their development and growth under salinity stress. This necessitates looking into the regulatory mechanisms that support the functions of H_2S as a stress-mitigating signaling molecule. Consequently, taking into account the significance of vegetable crops in the agricultural industry as well as their susceptibility to the NaCl stress, this study is focused on the exploration of the effect of H_2S via the use of its donor NaHS in NaCl-stressed sponge gourd and okra by considering its importance in abiotic stress mitigation.

Consequently, in this study, we have assessed how the H_2S donor NaHS influences the endogenous H_2S signaling and content, plant growth and development, pigment synthesis, carbohydrate content, protein content, antioxidants (enzymatic and non-enzymatic), and nitrogen metabolism in the presence of salt stress. The present study was conducted under controlled hydroponic conditions. Hydroponics was chosen to ensure uniformity, reproducibility, and precise control over salinity and exogenous NaHS levels, enabling accurate interpretation of plant responses without confounding soil-related factors. Besides, the hydroponic system is globally recognized and increasingly adopted in India, especially for salinity-affected or urban agriculture zones, making our study contextually and strategically relevant. Thus, the present study is aimed at exploring NaHS as an H_2S donor in improving the growth and development of *L. aegyptiaca* Mill, and *A. esculentus* under salinity stress with the following objectives:

1. Analysis of biomass accumulation pattern and status of photosynthetic pigments by growing vegetable crop under NaCl stress with and without exogenous NaHS.
2. Analysis of inorganic nitrogen contents and enzymes involved in nitrate and ammonia assimilation under NaCl stress with and without exogenous NaHS.
3. Study of reactive oxygen species, oxidative damage to lipids and proteins, and the role of antioxidants in withstanding the stress.
4. Understand the underlying signaling mechanism of H_2S in the test seedling during NaCl stress.

Chapter 2

2. REVIEW OF LITERATURE

2.1. The current state of soil salinity around the world

Soil salinization presents a significant global challenge to agricultural sustainability, affecting approximately 1.4 billion hectares of land, constituting 10.7% of the Earth's terrestrial surface (FAO, 2024). Plant growth and productivity are adversely affected by salty soils high in soluble salts or exchangeable sodium. Seventy percent of the world's salt-affected soils are currently found in ten countries, including China, Australia, Kazakhstan, Argentina, the United States, Russia, Sudan, Iran, Afghanistan, and Uzbekistan (FAO, 2024). Australia has the largest salt-affected soil among all nations.

India faces significant challenges related to land degradation, particularly due to urbanization and increased pressure on agricultural lands. Approximately 60% of India's geographical area is suitable for farming; 141 million hectares are used for crop production, while 10 million hectares are rangelands (Kumar and Sharma, 2020). The effects of soil degradation are considerably severe and potentially irreversible in semi-arid and arid regions. This presents a complex challenge for policymakers attempting to balance poverty eradication with food security goals.

The Food and Agriculture Organization reports that 2.95 million of India's 6.72 million hectares were classified as salty, while the remaining 3.77 million hectares were identified as sodic (Arora and Sharma, 2017; Kumar and Sharma, 2020). Gujarat is the state with the highest percentage of salt-affected soils (2.23 million hectares) in India, followed by Uttar Pradesh, Maharashtra, West Bengal, and Rajasthan (Mandal et al., 2018). Jaisalmer, Gujarat's coastline, and the Ganges basin constitute 20% of total salt- or sodicity-affected agricultural land. Whereas, Punjab constitutes 0.15 million hectares of salt-affected area, which is around 2.24% of the total salt-affected soil of India (CSSRI, 2025). The southwestern regions of Punjab, including Bathinda, Muktsar, Fazilka, Faridkot, Mansa, Sangrur and portions of the Bist-Doab area are affected by soil and groundwater salinity (Sharma et al., 2017; Dhaloiya et al., 2022; Singh et al., 2022). In India, the most widely planted crops on saline and sodic soils are millets, rice, cotton, barley, and sorghum. Maintaining viable farming in these areas demands focused approaches to soil and water resource management, implementation of salt-resistant crop varieties, and sustained environmental monitoring programs.

Annually, India loses approximately 1.5 million hectares of potential agricultural land to increasing soil salinity levels, significantly diminishing the nation's food production capacity (Atta et al., 2023). Anthropogenic activities and climate change are expected to escalate the area of salt-affected soils, with projections showing an increase from 6.72 to 16.2 million hectares by 2050 (CSSRI, 2015; Kumar and Sharma, 2020; Kumar et al., 2022). The estimated yearly economic loss in India due to soil salinity is around $US 3.0 billion, or 17 million metric tons of food grains. By the end of the next decade, this loss is predicted to rise to $US 5.6 billion (Kumar et al., 2022). These land degradation issues, particularly soil salinization, have far-reaching implications for environmental sustainability, agricultural productivity, livelihood security, and overall quality of life in affected regions (Kumar and Sharma, 2020). The situation demands immediate attention and implementation of effective management strategies to prevent further deterioration of agricultural lands.

2.2.Causes of salinity

There were two main causes of salinization: natural processes and human activities, as described in Fig. 2.1 (Kumar and Sharma 2020; Kumar et al., 2022). The natural factors that contribute to salinity include low rainfall, weathering, and rising sea level. By the end of the century, rising sea levels are expected to put over a billion people living in coastal areas at risk of progressive flooding and salinization. There has already been an eight- to nine-inch rise in the average sea level worldwide since 1880 (WMO, 2024; UNFCC, 2025).

Human-induced factors include irrigation practices, chemical fertilizers, poor crop management, and climate change. Over 17% of India's irrigated crops have been secondarily salinized because of brackish groundwater irrigation in arid and semi-arid regions, reducing agricultural productivity and soil fertility (Zaman et al., 2018; Singh et al., 2022; FAO, 2024). Salinity and sodicity contaminated 32-84% of groundwater resources across various Indian states, including Punjab, Rajasthan, Uttar Pradesh, Andhra Pradesh, Haryana, and Tamil Nadu (Choudhary & Bajwa, 2021; Kumar et al., 2022). Salinized areas expanded at an alarming rate of approximately 10% annually, driven by irrigation expansion and increased reliance on low-quality groundwater sources (Kumar and Sharma, 2020). Implementation of canal irrigation projects such

as the Sharda Sahayak Canal Project, Indira Gandhi Nahar Pariyojana, has significantly contributed to soil salinization, accounting for around 0.37 million hectares and 0.18 million hectares of land area, respectively (Kumar and Sharma, 2020; FAO, 2024).

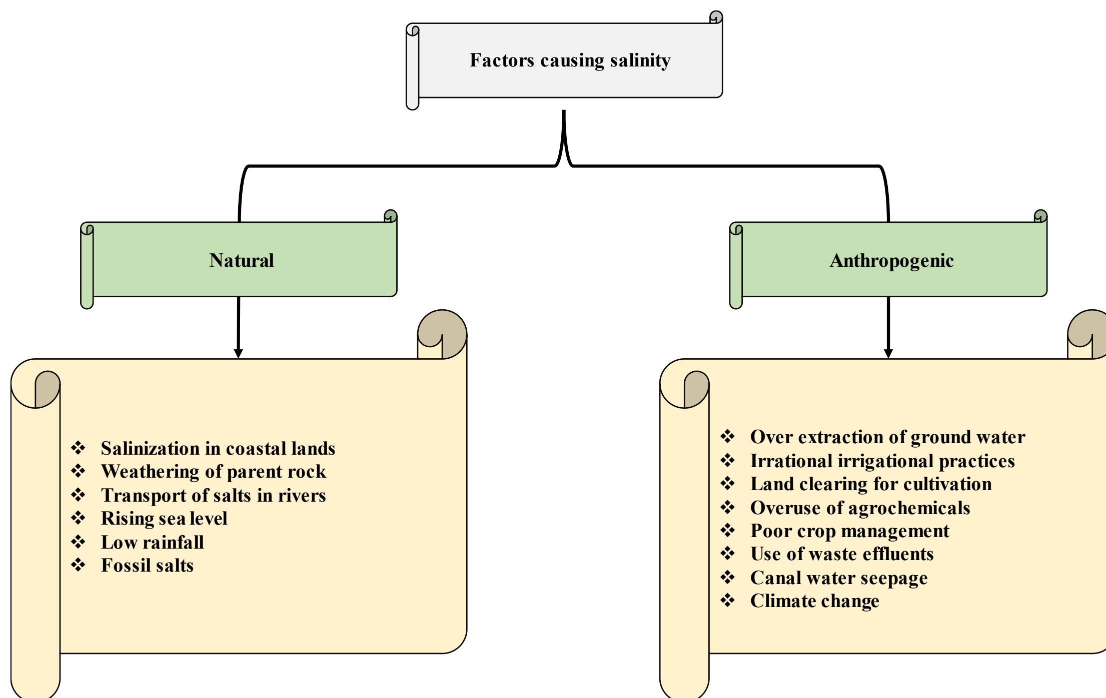

Figure 2.1. Factors affecting soil salinity.

The climate catastrophe is making aridity and freshwater scarcity worse. Global warming-induced permafrost thawing is also contributing to salinization. Poor farming methods significantly accelerate soil salinization through multiple mechanisms, including irrigation with subpar water, inadequate drainage implementation, widespread deforestation, aquifer overexploitation, excessive fertilizer application, use of de-icing agents, and mining operations. According to the Fertilizer Association of India, farmers' use of chemical fertilizers has skyrocketed by 95% between 2000 and 2021, hitting a record high of 32.5 million metric tons, due to unfavorable weather. Imperative agricultural resource conservation necessitates significant fertilizer reduction, as improper chemical application has precipitated extensive land degradation and aquatic eutrophication (Machado and Serralheiro, 2017). Critical challenges faced by modern agriculture must be overcome to sustain global food security.

2.3.Categorization of salinity

Salt content of soil is often represented by electrical conductivity (EC) (Kumar and Sharma, 2020). Whereas, other effective metrics for determining the risk of sodium in soil and soil solution are the Sodium Adsorption Ratio (SAR) and Exchangeable Sodium Percentage (ESP) (Atta et al., 2023). The United States Salinity Laboratory classified salt-affected soils into three categories, including saline, sodic, and saline–sodic (USSL 1954; Kumar et al., 2022).

Sodium, calcium, and magnesium sulphates and chlorides are frequently linked to the emergence of soil salinity (Atta et al., 2023). Saline soil (saturation phase pH (pHs)< 8.5, ESP< 15, saturation phase EC (ECe)> 4 dS m^{-1}) is mostly prevalent in arid or semi-arid areas due to low precipitation and rapid evaporation rates (Kumar and Sharma, 2020; Kumar et al., 2022). The saline soil tends to be dominated by water-soluble salts such as sulphates, chlorides, and carbonates (FAO, 2024).

Sodic soils (pHs> 8.5, ESP> 15, ECe< 4 dS m^{-1}) have a higher concentration of exchangeable sodium than other cations. Sodium bicarbonate ($NaHCO_3$) and sodium carbonate are the most prevalent salts of sodic soil (Kumar and Sharma, 2020; Kumar et al., 2022; Atta et al., 2023). These are typically distinguished by elevated dispersibility, excessive pH, and inadequate soil transmissibility (Atta et al., 2023). Whereas, saline-sodic soils are transitional between saline and sodic soils (pHs< 8.5, ESP> 15, ECe> 4 dS m^{-1}) (Kumar and Sharma, 2020; Kumar et al., 2022). The salt-affected soils in India are also classified using the same criteria as the USSL.

Salinity is categorized into primary and secondary salinity based on the sources that cause salinity. The former results from wind and rain carrying soluble salts from rock disintegration into soil. These salts mainy consist of chlorides of sodium, magnesium, and calcium, in addition to carbonates and sulphates (Kumar and Sharma, 2020; Giordano et al., 2021; Abdelaal et al., 2022). Whereas later is caused by human activities. These include switching from perennial to annual crops, using irrigation water with high salt concentrations, drawing too much groundwater, canal water seepage, and the excessive use of chemical fertilizers (Kumar and Sharma, 2020; Giordano et al., 2021; Abdelaal et al., 2022).

2.4. Effect of salt on growth attributes

2.4.1. Effect on germination and growth

Plants are vulnerable to salt stress, which reduces their growth and productivity by compromising essential physiological processes. Osmotic stress brought on by high salt concentrations first limits water absorption and quickly slows growth before ionic toxicity manifests (van Zelm et al., 2020; Guo et al., 2022). While plants experience harmful effects of salinity throughout their lifecycle, they remain most vulnerable during germination and early seedling development (Atta et al., 2023). The intricate, multi-phase developmental process of seed germination is influenced by salinity (Atta et al., 2023). Salinity reduces the osmotic potential of the germination medium, damages seed imbibition, causes ion toxicity, and causes nutritional imbalances, all of which prevent seeds from germinating (Munns et al., 2020; Lu et al., 2023). Additionally, salinity decreases α-amylase activity, critically impairing sugar translocation necessary for embryo development (Uçarli, 2020; Hasanuzzaman et al., 2021; Atta et al., 2023). These effects collectively delay and reduce germination rates. Salinity further reduces germination potential by changing the permeability of cell membranes, increasing the concentration of abscisic acid, and decreasing the concentration of gibberellic acid (Atta et al., 2023). Research consistently demonstrates a negative correlation between salinity and germination rates across species, including *S. bicolor* L. and *B. rapa* L. (Mulaudzi et al., 2020; Kamran et al., 2021; Chen et al., 2022). Viability of the embryo is compromised by chloride and sodium buildup even post-germination (EL Sabagh et al., 2021; Atta et al., 2023).

Vegetative growth also suffers significantly under salt stress, as shown in Table 2.1. Critical seedling characteristics, including fresh weight, root length, and shoot length, become impaired, serving as primary indicators of salt stress severity (Asif et al., 2020; Ahmed et al., 2022). Root functionality, density, and length are particularly compromised as salt accumulation on root surfaces disrupts water and nutrient absorption through root hairs (Siddiqui et al., 2017; Arif et al., 2019; Tanveer et al., 2020; Ahmed et al., 2022; Atta et al., 2023).

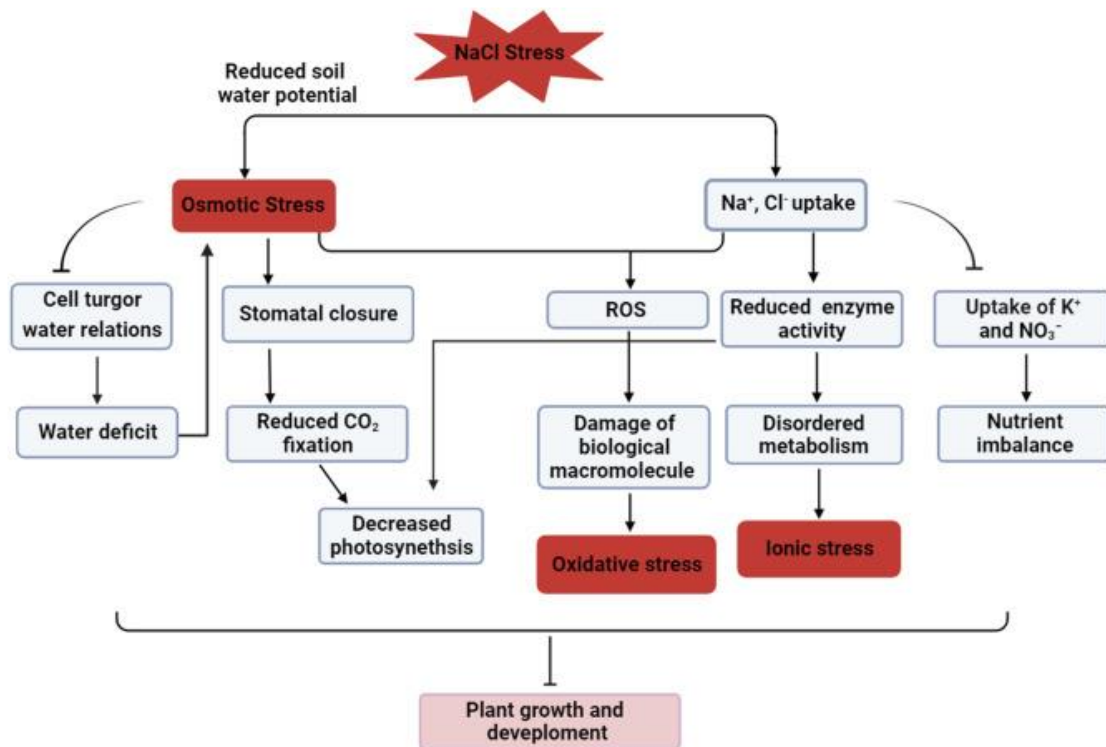

Figure 2.2. Depicts the detrimental consequences of salt stress on plants. Water deficiency and elevated intracellular sodium and chloride levels in saline environments are the primary causes of plant damage. Osmotic stress disrupts photosynthesis in plants by altering cell shape and causing stomatal closure. Plants that experience ionic stress due to high levels of sodium and chloride entering their cells experience decreased potassium-dependent enzyme activity, blocked nitrogen sources, metabolic abnormalities, and nutritional imbalance. ROS generation is also a result of osmotic and ionic stress, while oxidative stress ultimately affects plants by producing structural damage or altering the functional components of organelles. Collectively, these adverse effects hinder the normal development and growth of the plants (Zhou et al., 2024).

Salt damage operates through multiple pathways- dissolved salts in soil create osmotic effects that inhibit water uptake, diminish tissue water potential, and disrupt nutrient transport to shoots (Giordano et al., 2021; da Silva et al., 2022). Notably, roots exhibit earlier and more pronounced oxidative stress responses to salinity than leaf tissues (Guo et al., 2022). The plant's nutritional balance becomes disrupted through antagonistic effects on nutrient absorption and transport, reduced micronutrient solubility due to altered soil solution pH and redox potential (Giordano et al., 2021). These salinity-induced changes ultimately alter crucial processes, including photosynthesis and redox

balance, leading to declined growth and productivity of plants (Petretto et al., 2019; Giordano et al., 2021).

Table 2.1. Effects of NaCl stress on plant growth attributes.

Plant	**NaCl Stress Concentration (mM)**	**Outcome of the study**	**Reference**
C. annuum	100	Shoot, root, and total dry mass↓	Kaya et al., 2024
T. aestivum L.	100	Shoot and root length↓, plant height↓	Kumari et al., 2023
Solanum melongena and *S. lycopersicum* L.	20	Plant height↓	Raju and Prasad, 2023
S. italica L.	100	Plant height↓	Zhang et al., 2023b
S. lycopersicum L.	75 and 150	Plant height↓, plant and root fresh and dry weight↓	Yildirim et al., 2023
Helianthus annuus L.	150	Shoot and root length↓, fresh weight↓	Younis and Mansour, 2023
T. aestivum L.	100	Shoot length↓	Alamer, 2023
Brassica juncea L.	50, 100, and 150	Fresh weight↓, shoot, and root length↓	Verma et al., 2023a
Phaseolus vulgaris L.	50, 75, and 100	Fresh and dry biomass of shoot and root↓, plant height↓	Ekinci et al., 2023

Vigna radiata L.	50 and 100	Length and fresh weight of the shoot and root↓	Ullah et al., 2023
Moringa oleifera Lam.	50 and 100	Plant height↓, shoot biomass, and length↓	Azeem et al., 2023
T. aestivum L.`	30 and 60	Shoot length↓	Sadak et al., 2023
M. oleifera Lam.	100, 200, and 300	Length of root and shoot↓, fresh and dry weight↓	Abbas et al., 2023
Fagopyrum tataricum	100	Length of root and shoot↓, fresh weight↓	Zhang and Yang, 2022
S. bicolor L.	150	Seed germination↓, shoot, and root length↓	Chen et al., 2022
C. sativus L.	10	Root length↓	Liu et al., 2022a
P. vulgaris L.	75 and 150	Plant height↓, fresh weight of root and shoot↓	Dawood et al., 2022a
C. sativus L.	50 and 100	Fresh weight of root and shoot↓	Turan et al., 2022
O. sativa L.	100	Plant height↓, fresh weight of root and shoot↓, root length ↓	Wei et al., 2021b
B. rapa L.	200	Seed germination ↓, fresh weight ↓, radicle and plumule length ↓	Kamran et al., 2021
S. bicolor L.	200 and 300	Fresh weight ↓, germination percentage ↓, root and shoot length ↓	Mulaudzi et al., 2020

2.4.2. Salinity effect on pigment content

Photosynthetic pigments serve as reliable indicators of stress tolerance and effectively reveal both chloroplast development status and overall photosynthetic capacity in plants (Xue et al., 2013; Raju and Prasad, 2021). Salinity stress has an adverse effect on the pigment content of plants, and it reduces significantly due to the increment of chlorophyll-degrading enzyme (chlorophyllase) activity (Turan and Tripathy, 2015; Ahanger et al., 2019). Furthermore, chloride ions were found to interfere with the production of the pigment (Giordano et al., 2021). Salinity decreased carotenoid and xanthophyll levels, as well as chlorophyll fluorescence intensity, with chlorophyll b exhibiting greater sensitivity to increased salinity compared to chlorophyll a (Giordano et al., 2021). However, the content of total chlorophyll was found to decline with increasing concentration of NaCl stress (Guo et al., 2021; Yildirim et al., 2023; Azeem et al., 2023). The decline in the contents of chlorophyll a, chlorophyll b, and carotenoids under NaCl stress has been stated in *V. radiata* L., and the decline showed linearity with increasing NaCl concentrations (Ullah et al., 2023). *T. aestivum* L., *V. radiata* L., and *M. oleifera* Lam. exposed to 100 mM NaCl exhibited a decline in carotenoid, chlorophyll b, and chlorophyll a content (Alamer 2023; Ullah et al., 2023; Abbas et al., 2023). Whereas in *C. sativum, T. aestivum* L. under salinity, chlorophyll a and chlorophyll b content declined, but an enhancement in carotenoid content was reported (Kapoor et al., 2023; Sadak et al., 2023). A rise in alkalinity stress was shown to decrease chlorophyll SPAD values, likely resulting from elevated pH in the root. This higher pH interfered with both nutrient absorption and the production of photosynthetic pigments (Kaiwen et al., 2020; Loudari et al., 2022). Changes in plant photosynthetic efficiency impacted the synthesis of primary metabolites, including sugar and protein levels (Dawood and Azooz, 2019; Mostofa et al., 2020; Dawood et al., 2021). Soluble sugars, the primary photosynthetic products in plants, function as essential building blocks for macromolecular structures, and they perform a vital osmoregulatory function during salt stress (Nemati et al., 2011; Boriboonkaset et al., 2013). Dawood et al. (2021) suggested a strong correlation of plant photosynthetic pigments with soluble proteins and carbohydrates. Thus, a decline in pigment synthesis under salinity leads to a decline in carbohydrates and protein content.

The decrease in protein concentration, on the other hand, under NaCl stress can be explained by impaired nitrogen metabolism, ROS-induced protein degradation, and the detrimental effects of sodium and chloride ions on protein synthesis (Raju and Prasad, 2023). Reduced amount of chlorophyll production, photosynthetic rate, and carbon dioxide availability directly correlates to lower carbohydrate content in the leaves (Hassanein et al., 2009; Raju and Prasad, 2023). In *S. melongena* and *S. lycopersicum* L. under 20 mM of salinity stress, total chlorophyll, protein, and carbohydrate content showed reduction (Raju and Prasad, 2023). In *T. aestivum, B. juncea* L., chlorophyll and carbohydrate content were reduced on exposure to salinity stress of 100 mM and 50/100 mM, respectively (Sami et al., 2021; Kumari et al., 2023). Conversely, *C. sativum and Cynara scolymus* exhibited decreased pigment and protein levels, while showing increased carbohydrate content under stress (Dawood et al., 2021; Kapoor et al., 2023). The effect of salinity on these attributes has been observed in diverse crops, as shown in Table 2.2.

Table 2.2. The impact of NaCl stress on pigment, protein, and carbohydrate content in plants.

Plant	**NaCl Stress Concentration (mM)**	**Outcome of the study**	**Reference**
C. annuum	100	Chlorophyll a, and chlorophyll b content↓	Kaya et al., 2024
T. aestivum	100	Chlorophyll and carbohydrate content↓	Kumari et al., 2023
S. melongena and S. lycopersicum L.	20	Total chlorophyll, protein, and carbohydrate content↓	Raju and Prasad, 2023
S. italica L.	100	Chlorophyll a and b content↓, soluble sugar content↑	Zhang et al., 2023b
S. lycopersicum L.	75 and 150	Total chlorophyll, chlorophyll b, and chlorophyll a↓	Yildirim et al., 2023

T. aestivum L.	100	Carotenoid, chlorophyll b, and chlorophyll a↓	Alamer, 2023
B. juncea L.	50, 100, and 150	Protein content↓	Verma et al., 2023a
P. vulgaris L.	50, 75, and 100	Total chlorophyll, chlorophyll b, and chlorophyll a↓, sugar content↑	Ekinci et al., 2023
V. radiata L.	50 and 100	Protein content↑, Chlorophyll a, carotenoid, and chlorophyll b↓, soluble sugar content (first increase and then decrease).	Ullah et al., 2023
M. oleifera Lam.	50 and 100	Chlorophyll b and soluble sugar↑, chlorophyll a, and total chlorophyll↓	Azeem et al., 2023
T. aestivum L.	0, 30, and 60	Chlorophyll a, chlorophyll b↓, carotenoid content↑, carbohydrate content↓	Sadak et al., 2023
M. oleifera Lam.	100, 200, and 300	Chlorophyll and carotenoid content↓, protein content↓	Abbas et al., 2023
F. tataricum	100	Chlorophyll b, chlorophyll a, and chlorophyll (a, b) content↓, protein content↑	Zhang and Yang, 2022
S. bicolor L.	150	Sugar content↑, protein content↑	Chen et al., 2022
Lycopersicon esculentum Mill.	100	Protein content↓	Khan and AlZuaibr, 2022
C. sativus L.	10	Sugar content↓, protein content↓	Liu et al., 2022a

P. vulgaris L.	75 and 150	Chlorophyll b, carotenoid, chlorophyll a, and total pigment content↓	Dawood et al., 2022a
F. ananassa L.	15 and 30	Chlorophyll SPAD value↓	Bahmanbiglo and Eshghi, 2021
S. melongena and *S. lycopersicum* L.	20	Chlorophyll a, carotenoid, and chlorophyll b↓	Raju and Prasad, 2021
S. melogena	0, 50 and 100	Total chlorophyll content, chlorophyll b, and chlorophyll a↓	Ekinci et al., 2021
B. juncea L.	50 and 100	Chlorophyll SPAD value↓, carbohydrate content↓	Sami et al., 2021
B. rapa L.	200	Protein content↓, sugar content↑	Kamran et al., 2021
Thymus vulgaris L.	100	Chlorophyll b, carotenoid, chlorophyll a, and total chlorophyll↓	Zrig et al., 2021
Carthamus tinctorius L.	100, 200, and 300	Carotenoid content↓	Golkar and Taghizadeh, 2018

2.4.3. Salinity-induced oxidative stress and its impact on the antioxidant system

Salinity often causes an excessive accumulation of ROS, which are mostly generated in plant apoplasts, cytosol, endoplasmic reticulum, mitochondria, peroxisomes, and chloroplasts (Chen et al., 2021a; Atta et al., 2023). The stomatal closure brought on by elevated levels of cytoplasmic sodium ions damages the photosynthetic apparatus (Guo et al., 2022). Consequently, hydroxyl radicals, superoxide radicals, hydrogen peroxide, and singlet oxygen are produced in photosystem I by the Mehler reaction through excess energy transfer to oxygen molecules when the amount of light absorbed

surpasses the demand for photosynthesis (Petretto et al., 2019; Raza et al., 2020; Khorobrykh et al., 2020). Furthermore, in photosystem II, singlet oxygen and hydrogen peroxide are produced due to the restriction of electron movement between photosystems and the partial oxidation of water at the electron-donor side of photosystem II, respectively (Zhao et al., 2020). In addition to the chloroplasts, mitochondria, and peroxisomes also lead to significant ROS production. In mitochondria, superoxide radicals are primarily produced from molecular oxygen as a result of electron leakage from Complexes I and III of the electron transport chain, which is subsequently converted into hydrogen peroxide, while in peroxisomes, hydrogen peroxide is primarily produced (Liu et al., 2020b; Liu et al., 2021b).

ROS have been reported to play a dual function; at low concentrations, they engage in signalling, while at high concentrations, they become phytotoxic and cause oxidative stress (Hasanuzzaman et al., 2020a). ROS are exceedingly reactive and can induce oxidative damage by interacting with vital plant cell components, including DNA, lipids, proteins, and carbohydrates (Liu et al., 2020b; Moghanm et al., 2020; Saad-Allah et al., 2021; Abdelaal et al., 2022). Besides, they also induce enzyme inactivation, and hormonal and nutritional imbalances (Liu et al., 2020b; Hasanuzzaman et al., 2021). ROS damages photosynthetic pigments and induces cellular membrane deformities and dysfunction due to lipid peroxidation (Liu et al., 2020b). Malondialdehyde, a primary by-product of membrane lipid peroxidation in plants, serves as a key indicator of oxidative damage. Fluctuations in malondialdehyde levels and changes in plasma membrane permeability effectively gauge the severity of lipid peroxidation and membrane structural damage (Zhang and Yang, 2022). Prior studies have demonstrated that salt causes oxidative damage to a variety of crops, including *M. oleifera* Lam., *T. aestivum, and C. annuum* as categorized in Table 2.3 (Abbas et al., 2023; Kumari et al., 2023; Kaya et al., 2024).

In normal conditions, there exists a homeostasis between ROS production and scavenging, but during salinity stress, this coordination between production and scavenging of ROS gets disrupted because of an imbalance in the water potential of cells (Hasanuzzaman et al., 2020b). The inherent antioxidant system in plants helps them to regulate stress-mediated ROS production via enzymatic and non-enzymatic phytochemicals (Sies, 2020). Therefore, a corresponding increase in antioxidant

enzyme activity is observed in seedlings affected by salinity stress. Superoxide dismutase, catalase, and ascorbate peroxidase are several important antioxidant enzymes (Guo et al., 2022).

The first line of defense is superoxide dismutase, which transforms superoxide radicals into hydrogen peroxide. Ascorbate peroxidase, catalase, and glutathione peroxidase then detoxify hydrogen peroxide to water (Yang and Guo, 2018). Ascorbate peroxidase is another important enzymatic antioxidant in plants. It is a dynamic component of the ascorbate glutathione cycle and uses ascorbate as an electron donor to scavenge and convert hydrogen peroxide into water (Abdelaal et al., 2022). Many studies stated that ascorbate peroxidase is the most important and more active than other antioxidant enzymes in scavenging hydrogen peroxide under various stresses (Abdelaal et al., 2022). On the other hand, the process of hydrogen peroxide dismutation into water and oxygen is catalysed by catalase (Guo et al., 2022). The expression level of antioxidant enzymes has been reported in diverse plants under salinity stress (Table 2.3), including *C. annuum, C. sativum, H. annuus* L. (Younis and Mansour, 2023; Kapoor et al., 2023; Kaya et al., 2024).

Phenol and flavonoids are antioxidant compounds that act as cell-signalling agents (Michalak, 2006; Sadak et al., 2023; Abdelaal et al., 2022). Furthermore, they scavenge ROS and regulate the growth and development of plants under environmental stress (Hoang et al., 2020; Hu et al., 2022). The quantity and composition of phenols and flavonoids vary depending on the intensity of stress (de Abreu and Mazzafera, 2005; Azeem et al., 2023). Stress-induced physiological changes were found to increase phenol levels to mitigate the harmful impacts of salt stress (Abd El-Hameid and Sadak, 2020; Hu et al., 2022). Likewise, improvements in flavonoid levels during salinity may constitute a defense mechanism (Geetha et al., 2003; Sadak et al., 2023). Thus, the accumulation of phenols and flavonoids increases to counteract ROS under salt stress to shield plants from oxidative stress (Ghorbani et al., 2018; Santander et al., 2022). Additionally, these compounds shield the photosynthetic machinery from photo-oxidation by quenching free radicals and preventing the production of singlet oxygen (Niknam and Ebrahimzadeh, 2002; Agati, 2007; Azeem et al., 2023).

These compounds also offer protection from salt stress-related hazards like ultraviolet light, extreme heat, and drying out. Free radical scavenging capacity, measured through

2,2-diphenyl-1-picrylhydrazyl (DPPH) assays, serves as an important indicator of the antioxidant capabilities of phenolic compounds found in vegetable foods (Santander et al., 2022). The antioxidant activity (DPPH scavenging potential) was shown to be boosted by salinity in *T. aestivum* L., *M. oleifera* Lam., *C. tinctorius* L. to counteract ROS-induced oxidative damage (Golkar and Taghizadeh, 2018; Sadak et al., 2023; Azeem et al., 2023). Hence, the increment of enzymatic, non-enzymatic, and antioxidant potential of plants under stress occurs as a retaliation response to stress to prevent plants from oxidative damage.

Table 2.3. Represents the impact of NaCl stress on oxidative homeostasis and the antioxidant system.

Plant	NaCl Stress Concentration (mM)	Outcome of the study	Reference
C. annuum	100	Malondialdehyde↑, superoxide dismutase↑, hydrogen peroxide↑, catalase↓, ascorbate peroxidase↑	Kaya et al., 2024
T. aestivum L.	0, 30, and 60	Malondialdehyde↑, superoxide dismutase↑, hydrogen peroxide↑, catalase↑, phenol↑, flavonoids↑, DPPH↑	Sadak et al., 2023
M. oleifera Lam.	100, 200, and 300	Malondialdehyde↑, superoxide dismutase↑, catalase↑, phenol↓, flavonoids (variable decrease then increase), DPPH (variable decrease then increase)	Abbas et al., 2023
T. aestivum	100	Superoxide dismutase↑, hydrogen peroxide↑, ascorbate peroxidase↑	Kumari et al., 2023
S. italica L.	100	Superoxide dismutase↓, malondialdehyde↑, catalase↓, hydrogen peroxide↑	Zhang et al., 2023b

S. lycopersicum L.	75 and 150	Malondialdehyde↑, catalase↑, hydrogen peroxide↑, superoxide dismutase↑	Yildirim et al., 2023
H. annuus L.	150	Malondialdehyde↑, phenol↑, superoxide dismutase↑, DPPH↓, catalase↑, ascorbate peroxidase↑, hydrogen peroxide↑	Younis and Mansour, 2023
T. aestivum L.	100	Malondialdehyde↑, superoxide dismutase↑, phenol↑, catalase↑, hydrogen peroxide↑, ascorbate peroxidase↑	Alamer, 2023
B. juncea L.	50, 100, and 150	Hydrogen peroxide↑, malondialdehyde↑, phenol↓, flavonoids↓	Verma et al., 2023a
P. vulgaris L.	50, 75, and 100	Hydrogen peroxide↑, malondialdehyde↑, catalase↑, superoxide dismutase↑	Ekinci et al., 2023
V. radiata L.	50, and 100	Superoxide dismutase↑, hydrogen peroxide↑, ascorbate peroxidase↑, malondialdehyde↑, catalase (incr. at 50 mM and dec. at 100 mM), phenol ↑	Ullah et al., 2023
M. oleifera Lam.	50 and 100	Hydrogen peroxide↑, DPPH↑, malondialdehyde↑, total phenol↑, superoxide dismutase↑, flavonoids↑, catalase↑, ascorbate peroxidase↑	Azeem et al., 2023
C. sativus L.	10	Malondialdehyde↑, superoxide dismutase↑, hydrogen peroxide↑, catalase↑, ascorbate peroxidase↑	Liu et al., 2022a
P. vulgaris L.	75 and 150	Hydrogen peroxide↑, malondialdehyde↑, superoxide dismutase↑, ascorbate peroxidase↑, catalase↑	Dawood et al., 2022a

C. sativus L.	50 and 100	Catalase↑, hydrogen peroxide↑, superoxide dismutase↑, malondialdehyde↑	Turan et al., 2022
S. bicolor L.	150	Catalase↑, malondialdehyde↑, superoxide dismutase↑, hydrogen peroxide↑, ascorbate peroxidase↑	Chen et al., 2022
L. esculentum Mill.	100	Superoxide dismutase↑, hydrogen peroxide↑, catalase↑	Khan and AlZuaibr, 2022
F. tataricum	100	Malondialdehyde↑, superoxide dismutase↑, hydrogen peroxide↑, catalase ↓	Zhang and Yang, 2022
B. rapa L.	200	Catalase↓, hydrogen peroxide↑, superoxide dismutase↑, malondialdehyde↑	Kamran et al., 2021
T. vulgaris L	100	Catalase↑, hydrogen peroxide↑, superoxide dismutase↑, malondialdehyde↑, ascorbate peroxidase↑	Zrig et al., 2021
O. sativa L.	100	Superoxide dismutase↑, hydrogen peroxide↑, ascorbate peroxidase↑, malondialdehyde↑, catalase↓	Wei et al., 2021b
F. ananassa L.	15 and 30	Hydrogen peroxide↑, malondialdehyde↑, ascorbate peroxidase (variable), superoxide dismutase (variable)	Bahmanbiglo and Eshghi, 2021
Cyclocarya paliurus	100	Catalase↑, malondialdehyde↑, ascorbate peroxidase↑, hydrogen peroxide↑, superoxide dismutase↑	Chen et al., 2021b
S. melongena and *S. lycopersicum* L.	20	Catalase↑, hydrogen peroxide↑, superoxide dismutase↑, malondialdehyde↑	Raju and Prasad, 2021

S. melogena	50 and 100	Catalase↑, hydrogen peroxide↑, superoxide dismutase↑, malondialdehyde↑	Ekinci et al., 2021
B. juncea L.	50 and 100	Catalase↑, hydrogen peroxide↑, superoxide dismutase↑, malondialdehyde↑	Sami et al., 2021
C. tinctorius L.	100, 200, and 300	Malondialdehyde↑, phenol↑, flavonoid↑, DPPH↑	Golkar and Taghizadeh, 2018
A. thaliana	150	Malondialdehyde↑, superoxide dismutase↓, catalase↓	Yastreb et al., 2020

2.4.4. Salinity and nitrogen metabolism

Nitrogen is a vital plant nutrient as it is a part of numerous plant compounds, including proteins, polyamines, amino acids, quaternary ammonium compounds, and amides. These nitrogen-containing compounds contribute to plant salt tolerance through multiple mechanisms, particularly osmotic regulation and ROS detoxification (Zaki 2016; Arghavani et al. 2017; Ashraf et al., 2018). Therefore, the tolerance of salinity in plants depends critically on nitrogen metabolism (Daniel-Vedele et al., 2010). The metabolism of nitrogen in crop plants relies on several key enzymes, including nitrate and nitrite reductase, along with glutamine synthetase, glutamate synthase, and glutamate dehydrogenase (He et al. 2022; Rizwan et al. 2022). The activity levels of these enzymes serve as precise indicators of the extent of nitrogen metabolism in crops (Xia et al. 2020).

The primary nitrogen forms in plant systems consist of nitrate, nitrite, and ammonia (Yuan et al., 2007; Tian et al., 2022). Plants take up nitrogen in the form of nitrate and ammonia from the soil. Nitrate undergoes reduction into ammonia via nitrate reductase and nitrite reductase inside the plant cell Yoneyama and Suzuki 2019; Liu et al., 2021c; Tian et al., 2022). The ammonia is then assimilated into organic compounds via a two-step reaction, first into glutamine in the presence of glutamine synthetase, which is then converted into glutamate by the action of glutamate synthase (Fortunato et al., 2023).

The activities of glutamine synthetase and glutamate synthase indicate the level of amino acid synthesis, which subsequently supports macromolecular compound formation (Lam et al., 1996; Cañas et al., 2020; Sahay et al., 2021).

Under saline conditions, plants experience significant disruptions in nitrogen uptake and metabolism. This occurs through several mechanisms: the inhibition of nitrate, ammonia, and water uptake (Ehlting et al., 2007; Ashraf et al., 2017), antagonistic effects between chloride and nitrate ions (Giordano et al., 2021), and the inactivation of nitrate transporters such as OsNRT1 (Balliu et al., 2015). In addition, the activities of key nitrogen metabolism enzymes are sensitive to salt stress (Du et al., 2017). Salt stress further impairs nitrogen utilization by disrupting the loading of nitrogen ions into root xylem (Baki et al., 2000) and damaging root membrane integrity (Debouba et al., 2007). Additionally, plants under salinity stress often have reduced growth rates, leading to lower nitrogen demand (Ullrich, 2001). The stress response also triggers hydrolyzing enzymes, such as DNase, RNase, and protease (Debouba et al., 2007; de Souza et al., 2016).

Furthermore, under salinity ammonia content gets elevated due to enhanced proteolysis (Wang et al. 2007). The elevated ammonia levels induce aminating glutamate dehydrogenase, causing a shift in the nitrogen assimilation pathway from the glutamine synthetase-glutamate synthase (GS-GOGAT) route to the glutamate dehydrogenase-mediated pathway (Ashraf et al., 2018). Under salt stress conditions, glutamate dehydrogenase performs a specialized physiological function by eliminating excess ammonia (Peleg and Blumwald, 2011). In the glutamate dehydrogenase pathway, ammonia can be directly converted to glutamate by glutamate dehydrogenase (Ashraf et al., 2018). This shift in pathway has speculated the involvement of glutamate dehydrogenase in ammonia detoxification and glutamate replenishment in vivo, thus promoting the production of protective metabolites such as proline, phytochelatines, etc. Elevated intracellular ammonia accumulation during salinity exerts cytotoxic effects, resulting in multiple cellular disruptions, including pH imbalance, osmotic dysregulation, inhibition of ATP production, nutritional deficiencies, and tissue necrosis. Plants in which the effect of salinity on nitrogen metabolism was reported are listed in Table 2.4, and it includes *T. aestivum* L. (Kumari et al., 2023; Alamer, 2023;

Sadak et al., 2023), *S. melongena* and *S. lycopersicum* L. (Raju and Prasad, 2023), and *V. radiata* L. (Ullah et al., 2023).

Table 2.4. Impact of NaCl toxicity on inorganic nitrogen content and nitrogen metabolism enzymes.

Plant	**NaCl Stress Concentration (mM)**	**Outcome of the study**	**Reference**
T. aestivum	100	Nitrogen content↓, nitrate reductase↓	Kumari et al., 2023
S. melongena and S. lycopersicum L.	20	Nitrate↓, nitrite↓, ammonia↑, glutamate synthase↓, nitrate and nitrite reductase↓, glutamate dehydrogenase↑, glutamine synthetase activity↓	Raju and Prasad, 2023
T. aestivum L.	100	Nitrate reductase↓	Alamer, 2023
T. aestivum L.	0, 30, and 60	Nitrate reductase↑	Sadak et al., 2023
V. radiata L.	50 and 100	Nitrogen content↓, nitrate reductase↓, glutamine synthetase↓, glutamate synthase↓	Ullah et al., 2023
L. esculentum Mill.	100	Nitrate reductase↑, nitrite reductase↑, glutamine synthetase↓, glutamate synthase↓, glutamate dehydrogenase↓, nitrate (non-significant effect), nitrite↓, ammonia↑, nitrogen content↓	Khan and AlZuaibr, 2022
P. vulgaris L.	75 and 150	Nitrate reductase↑	Dawood et al., 2022a
C. paliurus	100	Nitrate reductase↑	Chen et al., 2021b

B. juncea L.	50 and 100	Nitrate reductase↓	Sami et al., 2021
Heavy metal stress			
O. sativa L.	0,50,100,200 µM (Nickel conc.)	Nitrate↓, nitrate and nitrite reductase↓, ammonia↑, glutamine synthetase↓, glutamate dehydrogenase↑, glutamate synthase↓	Rizwan et al., 2022
Cucurbita pepo	50 and 100 mg L^{-1} (Nickel)	Nitrate reductase ↓	Valivand and Amooaghaie, 2021b
O. sativa L.	200 µM (Nickel)	Nitrate↓, nitrate and nitrite reductase↓, ammonia↑, glutamine synthetase↓, glutamate dehydrogenase↑, glutamate synthase↓	Rizwan et al., 2019
Vicia faba L.	5 µM (Arsenic)	Nitrate and nitrite reductase↓, glutamine synthetase↓	Siddiqui et al., 2021
Drought stress			
C. annuum	40% of field capacity	Nitrate and nitrite reductase↓, glutamate synthase↓, glutamine synthetase↓, ammonia↑, glutamate dehydrogenase↓, nitrate↑, total nitrogen↓	Kaya and Shabala, 2023

2.5. H_2S as a stress modulator

The environmental challenges hamper agricultural productivity by impacting plant growth, maturation, and performance (He et al., 2018). Reduced fertility affects an estimated 40% of the world's arable land, and rising demands are driving up infertility, thereby subjecting the flora and fauna diversity to risk (Cristofano et al., 2021). Reducing these losses is a key challenge to maintain food security in light of the world's

changing environment and expanding population, whilst reducing or minimizing the environmental impact. Therefore, different stress mitigation strategies have been explored, one among which is the supplementation of exogenous growth promoters. H_2S, is among the various signaling molecules that significantly regulate plant stress responses via a cascade of biochemical and physiological changes across plant cells (Wang et al., 2012; Corpas et al., 2019; Wang et al., 2021). It acts as a substantial secondary messenger that modulates both stress responses and developmental processes in plant cells under abiotic stressors (Khan et al., 2017; Corpas et al., 2019; Raju and Prasad, 2021).

H_2S has been extensively researched for its ability to regulate many biochemical, physiological, and cellular processes in plants under stress, as represented in Table 2.5 (Guo et al., 2018; Hasanuzzaman et al., 2018; Karle et al., 2021). H_2S is endogenously biosynthesized in mitochondria, chloroplasts, and cytosol of plant cells by sulphur metabolism (Aroca et al., 2018; Corpas et al., 2019; Chen et al., 2020). The principal enzymes in H_2S biogenesis are L-cysteine desulfhydrase (DES1), D-cysteine-desulfhydrase (DCD), O-acetylserine (thiol) lyase (OAS), L-cysteinedesulfurase (DSF), sulfite-reductase (SiR), β-Cyanoalanine synthase (CAS-C1), and adenosine phosphosulfate (APS), (Yamasaki and Cohen, 2016; Khan et al., 2018; Yamasaki et al., 2019; Li et al., 2019; Vojtovič et al., 2021; Dai et al., 2024).

Among these, SiR is the dominant enzyme of H_2S biogenesis, which is located in the chloroplast. It catalyzes the synthesis of H_2S during the sulfate assimilation in the presence of cofactor ferrodoxin (Fd) (Arif et al., 2021). DES1 is located in the cytosol and it synthesizes H_2S during cysteine (Cys) degradation with the help of a cofactor pyridoxal phosphate (PLP) (Alvarez et. al. 2010; Kaya and Ashraf, 2020; Kaya et. al. 2020). The enzyme CAS-C1 is a mitochondrial-bound enzyme synthesizing H_2S during cyanide (CN^-) detoxification with cofactor PLP and also from Cys (Gotor et al., 2019; Yamasaki et al., 2019; Arif et al., 2021; Vojtovič et al., 2021). Besides this, other enzymes, including OAS and Nifs-like proteins, also contribute to H_2S biosynthesis (Gotor et al., 2019).

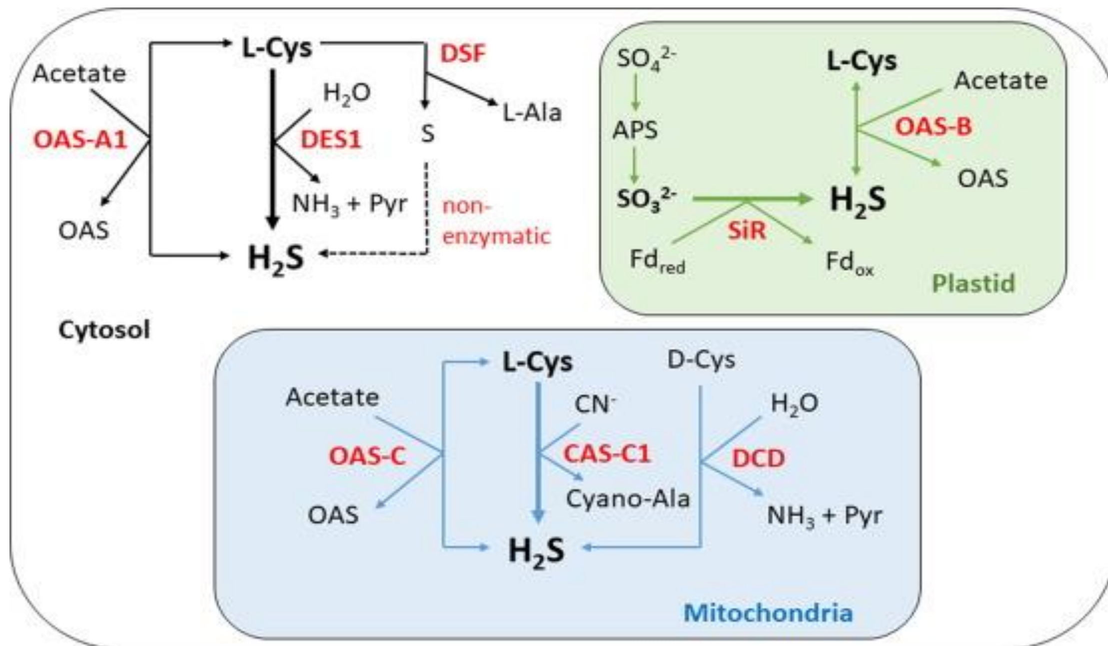

Figure 2.3. An outline of endogenous H_2S synthesis in plants (Vojtovič et al., 2021).

Endogenous H_2S concentration was observed to be upregulated under various stress circumstances in different plants, including *C. annuum* (Kaya and Shabala, 2023; Kaya et al., 2024), *C. sativum (*Kapoor et al., 2023), *S. italica* L. (Zhang et al., 2023b), *H. annuus* L. (Younis and Mansour, 2023), *T. aestivum* L. (Ding et al., 2019; Alamer, 2023), *P. vulgaris* L. (Dawood et al., 2022a), *S. melongena* (Raju and Prasad, 2021), *V. faba* L. (Siddiqui et al., 2021), *Cucurbita pepo* (Valivand and Amooaghaie, 2021a), and *L. esculentum* Mill. (Khan and AlZuaibr, 2022; Wu et al., 2024)

H_2S mediates rescue of salinity-stressed seedlings by preserving ion homeostasis, osmotic, and oxidative equilibrium (Mostofa et al., 2015a; Younis and Mansour, 2023; Alamer, 2023). Ion homeostasis is preserved through the modulation of the expression of sodium/hydrogen antiporter (Na^+/H^+-antiporter) protein and plasma membrane-localized H^+-ATPase, which work together to promote sodium ion extrusion and restrain sodium ion uptake (Deng et al., 2016; Jiang and Tian, 2019; Wei et al., 2022). Besides, it inhibits the potassium ion efflux by regulating the outward rectifying shaker-type potassium channel (Deng et al., 2016; Jiang and Tian, 2019). Plasma membrane-bound H^+-ATPase establishes an H^+ gradient (Blumwald et al., 2000), which is used by Na^+/H^+-antiporter that is salt overly sensitive 1 to expel sodium ions outside of the cell cytoplasm to preserve ionic homeostasis (Flowers et al., 1977).

Besides regulating ion homeostasis, H_2S also regulates seedling growth during salinity stress by counterbalancing the deleterious effects of oxidative stress by up-regulating ROS scavenging, enzymatic, and non-enzymatic antioxidants. The enzymatic antioxidants include the enzymes of the ascorbate glutathione cycle, superoxide dismutase, and catalase. The ascorbate glutathione cycle enzymes include mono-dehydro-ascorbate reductase, ascorbate peroxidase, glutathione reductase, and dehydro-ascorbate reductase. They help to regulate cellular homeostasis by scavenging ROS like hydroxyl radicals, superoxide radicals, and hydrogen peroxide, thus minimizing lipid peroxidation, DNA damage, and electrolyte leakage (Chen et al., 2017; Kaya et al., 2018; Corpas et al., 2019; Ding et al., 2019; Younis and Mansour, 2023). Besides, H_2S also upregulates the antioxidant capacity to promote stress endurance in plants by regulating the non-enzymatic antioxidants (phenol and flavonoid) (Dawood et al., 2021; Valivand and Amooaghaie, 2021a; Younis and Mansour, 2023; Verma et al., 2023a). H_2S also prevents the pigment, protein, and carbohydrate damage from the salinity stress (Kapoor et al., 2023; Raju and Prasad, 2023). Furthermore, H_2S has been reported to regulate the nitrogen metabolism under stress in diverse crops including *C. annuum*, *S. melongena*, *S. lycopersicum* L., *V. faba* L., and *O. sativa* L. by regulating the nitrogen uptake and assimilation through modulating the expression of nitrogen metabolism enzymes (nitrite and nitrate reductase, glutamate synthase, glutamate dehydrogenase, and glutamine synthetase) (Rizwan et al., 2019; Siddiqui et al., 2021; Kaya and Shabala, 2023; Raju and Prasad, 2023). From the table 2.5. it can be concluded that earlier studies on vegetable crops such as *C. annuum*, *P. vulgaris* L., *S. melongena and S. lycopersicum* L., and *C. sativus* L. have consistently demonstrated the beneficial role of H_2S in alleviating salinity stress (Kaya et al., 2024; Ekinci et al., 2023; Raju and Prasad, 2023; Yildrim et al., 2023; Dawood et al., 2022a; Raju and Prasad, 2021; Ekinci et al., 2021; Liu et al., 2022a; Turan et al., 2022; Jiang et al., 2019). Altogether, from the findings of these studies, it can be inferred that vegetable species consistently exhibit improved growth, enhanced pigment content, and strengthened antioxidant machinery in response to H_2S exposure under a saline environment. Hence, it becomes important to understand the mechanism and role of H_2S during plant stress mitigation. The application of H_2S appears to be an

effective and sustainable approach for improving crop performance under a salinity stress environment.

Table 2.5. Represents the salt stress mitigating potential of H_2S in plants.

Plant Type	**Type of stress**	**Stress Concentration(mM)**	**Concentration of H_2S donor** (µM)	**Outcome of the study**	**References**
C. annuum	Salinity	100	200	Total, root and shoot dry mass↑, H_2S↑, chlorophyll a↑, hydrogen peroxide↓, chlorophyll b↑, malondialdehyde↓, superoxide dismutase↓, catalase↑, ascorbate peroxidase↑	Kaya et al., 2024
S. melongena and S. lycopersicum L.	Salinity	20	40	Plant height↑, total chlorophyll↑, nitrate↑, nitrite↑, ammonia↓, nitrate and nitrite reductase↑, glutamate synthase↑, glutamate dehydrogenase activity↓, protein content↑, glutamine synthetase↑, carbohydrate content↑	Raju and Prasad, 2023
S. italica L.	Salinity	100	200	Plant height↑, H_2S↑, malondialdehyde ↓, catalase↑, chlorophyll (a,b)↑, hydrogen peroxide↓, soluble sugar↓, superoxide dismutase↑	Zhang et al., 2023b
S. lycopersicum L.	Salinity	75 and 150	25, 50, 75, and 100	Plant fresh and dry weight↑, total chlorophyll↑, plant height↑, chlorophyll a↑, root fresh and dry weight↑, chlorophyll b↑,	Yildirim et al., 2023

				hydrogen peroxide↓, malondialdehyde↓, catalase↓, superoxide dismutase↓	
T. aestivum L.	Salinity	100	20 and 50	Shoot length↑, carotenoid↑, chlorophyll a↑, hydrogen peroxide↓, chlorophyll b↑, malondialdehyde↓, nitrate reductase↑, superoxide dismutase↑, phenol↑, catalase↑, H_2S↓, ascorbate peroxidase↑	Alamer, 2023
B. juncea L.	Salinity	50, 100, and 150	25	Root length↑, shoot length↑, fresh weight↑, malondialdehyde↓, hydrogen peroxide↓, flavonoid↑, phenol↑, protein↑	Verma et al., 2023a
P. vulgaris L.	Salinity	50, 75, and 100	25, 50, 75, and 100	Shoot fresh and dry mass↑, root fresh and dry mass↑, plant height↑, total chlorophyll↑, sugar↓, chlorophyll a↑, malondialdehyde↓, chlorophyll b↑, hydrogen peroxide↓, catalase↓, superoxide dismutase↓	Ekinci et al., 2023
H. annuus L.	Salinity	150	500	Fresh weight↑, shoot and root length↑, hydrogen peroxide↓, DPPH↑, malondialdehyde↓, H_2S↑, superoxide dismutase↓, phenol↓, catalase↓, ascorbate peroxidase↑	Younis and Mansour, 2023
C. sativus L.	Salinity	10	100	Root length↑, sugar↑, protein↑, malondialdehyde↓, hydrogen peroxide↓,	Liu et al., 2022a

				superoxide dismutase↑, ascorbate peroxidase↑, catalase↑	
P. vulgaris L.	Salinity	75 and 150	50 and 100	Plant height↑, shoot and root fresh weight↑, total pigment content↑, chlorophyll a↑, malondialdehyde↓, chlorophyll b↑, hydrogen peroxide↓, H_2S↑, carotenoid↑, catalase↑, superoxide dismutase↑, ascorbate peroxidase↑, nitrate reductase↑	Dawood et al., 2022a
C. sativus L.	Salinity	50 and 100	25, 50, 75, and 100	Shoot fresh and dry weight↑, hydrogen peroxide↓, root fresh weight↑, malondialdehyde↓, catalase↓, superoxide dismutase↓	Turan et al., 2022
A. marina	Salinity	400	200	Salt secretion↑, H_2S content↑, Na^+/K^+ ratio↑, Na^+/K^+ antiporter↑, H^+-ATPase↑	Wei et al., 2022
O. sativa L.	Salinity	100	100	Plant height↑, root and shoot fresh weight↑, hydrogen peroxide↓, root length↑, superoxide dismutase↓, malondialdehyde↓, catalase↑, ascorbate peroxidase↑	Wei et al., 2021b
S. melongena and *S. lycopersicum* L.	Salinity	20	40	Fresh and dry weight↑, length of shoot and root↑, H_2S↑, carotenoid↑, chlorophyll a↑, hydrogen peroxide↓, chlorophyll b↑, malondialdehyde↓,	Raju and Prasad, 2021

				catalase↑, superoxide dismutase↑	
S. melongena	Salinity	50 and 100	25, 50, and 100	Plant height↑, fresh weight↑, chlorophyll a↑, chlorophyll b↑, total chlorophyll↑, hydrogen peroxide↓, malondialdehyde↓, catalase↓, superoxide dismutase↓	Ekinci et al., 2021
F. ananassa L.	Alkalinity	0,15 and 30	200 and 500	Shoot dry weight↑, root dry weight↑, chlorophyll SPAD value↑, ascorbate peroxidase (variable)↓, superoxide dismutase (variable)↓	Bahmanbiglo and Eshghi, 2021
C. paliurus	Salinity	100	500	Total biomass↑, nitrate reductase↑, hydrogen peroxide↓, ascorbate peroxidase↑, malondialdehyde↓, catalase↑, superoxide dismutase↑	Chen et al., 2021b
A. thaliana	Salinity	150	50	Total chlorophyll↑, carotenoid↑, malondialdehyde↓, superoxide dismutase↑, catalase↑, sugar↑	Yastreb et al., 2020
C. sativus L.	Salinity	200	5, 10, 15, and 20	Fresh biomass↑, length of root and shoot↑, total chlorophyll↑, carotenoid↑, malondialdehyde↓, chlorophyll a↑, hydrogen peroxide↓, chlorophyll b↑, H_2S↑, superoxide dismutase↓, catalase↑	Jiang et al., 2019
Kandelia obovate	Salinity	400	200	Total chlorophyll↑, hydrogen peroxide↓,	Liu et al., 2019b

				superoxide dismutase↑, ascorbate peroxidase↑	
T. aestivum	Salinity	160	50	Plant height↑, chlorophyll↑, hydrogen peroxide↓, H_2S↑, malondialdehyde↓, catalase↑, superoxide dismutase↑	Ding et al., 2019
M. hupehensis	Salinity	85	50	Root length↑, hydrogen peroxide↓, superoxide dismutase↑, catalase↑	Wei et al., 2019

2.6.Endogenous H_2S dynamics in plants: Basal level and stress responses

Under normal growth conditions, endogenous H_2S content or production varies significantly across plant species. Measurements range from as low as 2 nmol g^{-1} fresh weight to as high as 7 µmol g^{-1} fresh weight, or 0.38 to 6 nmol mg^{-1} protein min^{-1}. These considerable variations likely stem from differences in measurement methodologies, plant species characteristics, developmental stages, and experimental systems employed in various studies (Li et al., 2016).

During abiotic stress exposure, plants typically exhibit a significant elevation in endogenous H_2S concentrations, with levels increasing approximately 2-2.5 times across diverse plant species (Li et al., 2016). This consistent upregulation signifies that H_2S is a secondary messenger that detects stress and initiates physiological responses through H_2S-mediated signaling (Lai et al., 2014). This stress-induced H_2S elevation may serve as a critical molecular trigger that initiates cross-adaptation mechanisms in plants, enabling them to develop resilience against multiple environmental challenges through interconnected defense responses.

In recent times, H_2S-intervened complex signaling has been drawing the attention of plant biologists worldwide. To completely comprehend and exploit the functions of H_2S in agricultural growth and stress resilience, H_2S signaling pathways need to be explored and revealed.

2.7.Atmospheric H_2S and the need for exogenous H_2S donors

H_2S is naturally produced by anaerobic bacteria during decomposition of organic sulfur compounds in wetlands, estuaries, salt marshes as well as from geothermal vents and volcanoes (Watts, 2000; Stern, 2005). In addition, anthropogenic activities such as industrial activities, livestock rearing, and burning of fossil fuels and biomass also contribute to its production (Watts, 2000). The absorption of H_2S by plants follows Michaelis-Menten kinetics and is regulated by mesophyll conductance (De kok et al., 1997; Ausma and De kok, 2019). In mesophyll cells with pH 5-6.4, the H_2S predominantly exists in its undissociated form, with a Henry's law solubility constant of 0.086 M/atm at 25°C ($H_2S \rightarrow HS^- + H^+$; pKa=7) (Riahi and Rowley, 2014). Moreover, H_2S, being lipophilic, moves freely across the lipid bilayer without the need for transporters (Cuevasanta et al., 2017). Once inside the cell, it is incorporated into cysteine and also contributes to endogenous H_2S synthesis. The general low endogenous quantity of H_2S makes it challenging to identify its specific biological role, necessitating exogenous donors to increase endogenous H_2S concentrations sufficiently to trigger physiological responses (Powell et al., 2018).

H_2S is most easily applied as a gas; however, this is not practicable outside of the laboratory for safety reasons (Rubright et al., 2017). Consequently, a variety of donor molecules have been used to supplement H_2S, and numerous studies have been conducted to investigate its physiological and signaling properties to exploit its potential therapeutic advantages, as represented in Table 2.5.

The susceptibility to H_2S varies greatly throughout species and even amongst different cultivars within the same species. Retarded growth has been documented in certain plant seedlings (spinach) exposed to 0.03 μLL^{-1} H_2S in industrially polluted areas, however, the same dose promoted growth of other plants in sulfur-deficient soils (Thompson and Kats, 1978; De Kok, 1990). *Miscanthus sinensis,* a species found near volcanic areas, can tolerate H_2S concentrations as high as 20 µL/L (Mudd, 1979). The variation in plant sensitivity to H_2S is dependent on the way it affects energy balance in plants (Ausma and De kok, 2019).

2.8.Donors of H_2S

Compounds that release H_2S are called H_2S donors which include NaHS, dialkyl-dithio-phosphates, calcium sulfide, morpholin-4-ium4-methoxy-phenyl-(morpholino)-phosphinodithioate (GYY4137), AP39, diallyl trisulfide, and NOSH-aspirin (Huang et al., 2021). The rapid hydrolysis of NaHS in water results in an immediate equilibrium of H_2S, hydrogen sulfide ions (HS^-), and sulfide ion (S^{2-}), making it a useful tool in plant study (Powell et al., 2018). On the other hand, NaHS releases H_2S directly and instantly in contrast to GYY4137 (Li et al., 2008; Thakur and Anand, 2021). GYY4137 is the first known small organic H_2S donor that releases H_2S via hydrolysis (Li et al., 2008). However, H_2S release from GYY4137 occurs slowly and over an extended duration, resulting in more pronounced biological study conditions characterized by longer exposure times and reduced H_2S shock (Whiteman et al., 2015). Applying both GYY 4137 and NaHS to *C. annuum*, Lisjak et al. (2011) found that GYY 4137 caused a delayed release of H_2S compared to NaHS. Therefore, NaHS is usually preferred as an H_2S donor.

2.9.Mechanism of H_2S release from NaHS

In plant-related research, NaHS is sprayed directly onto plants or added exogenously to hydroponic solution or in vitro growth substrate. The donor NaHS has a brief lifespan (Corpas and Palma, 2020) and rapidly dissociates in aqueous solutions to release H_2S (Zhao et al., 2024; Ahmed et al., 2021; Whiteman et al., 2010). When in solution, it hydrolyzes to form sodium ions (Na^+) and HS^-. The HS^- ions then react with hydrogen ions (H^+) in the solution, resulting in instant release of H_2S (Hosoki et al., 1997; Thakur and Anand, 2021). Moreover, in the solution, there exists an equilibrium between HS^-, S^{2-}, and H_2S (Chen and Morris, 1972; Magli et al., 2021). In physiological circumstances and biological fluids, H_2S is largely undissociated (Caliendo et al., 2010).

Due to its gaseous nature and lipophilicity, H_2S exhibits high membrane permeability (Riahi and Rowley, 2014; Li et al., 2016; Thakur and Anand, 2021), facilitating easy penetration into seeds and plants via simple diffusion to regulate physiological functions by stimulating endogenous H_2S content (Li et al., 2016; Thakur and Anand, 2021). Several other studies have reported that exposure of seedlings to exogenous

NaHS increases the activity of H_2S-synthesizing enzymes, including DES1, DCD, OAS, and CAS-C1, thereby elevating endogenous H_2S content (Khan et al., 2018; Li et al., 2019; Dai et al., 2024).

2.10. Rationale for investigating H_2S potential in NaCl stress mitigation (Hypothesis)

In recent times, because of the excessive use of fertilizers, irrational irrigational practices, and climate change, the salinity content in soil is increasing at an alarming rate, posing a significant challenge to agricultural productivity. The salt-affected soils present an escalating global agricultural challenge that threatens food production. This challenge necessitates the urgent development of effective methods to improve crop resistance to saline conditions. Thus, diverse types of research are being conducted to find ways that can abate the adverse effects of salinity to sustain food security for an expanding global population.

From the above literature, understandably, the evolving research in the field of gaseous transmitters suggests H_2S as a multifaceted signaling molecule in plant stress responses. As a gaso-transmitter, H_2S possesses unique properties that enable it to influence multiple cellular processes simultaneously, including antioxidant defense activation, osmotic regulation, and ion homeostasis maintenance, all critical factors in salt stress tolerance (Zhang et al., 2020; Siddiqui et al., 2021; Jiang et al., 2019; Younis and Mansour, 2023; Khan et al., 2021). Its ability to interact with plant hormones and modify proteins through S-sulfhydration provides additional layers of regulation that may orchestrate comprehensive salt stress responses (Kolupaev et al., 2022; Filipovic, 2015; Paul and Snyder, 2015; Zhang et al., 2017). H_2S consistently shows a 2-2.5fold increase under various abiotic stresses, suggesting its fundamental role in stress adaptation mechanisms (Shi et al., 2015; Li et al., 2016; Santisree et al., 2019; Bhuyan et al., 2020). Thus, investigating H_2S as a potential mitigator of NaCl stress in plants represents a promising research direction with significant scientific and agricultural implications.

Despite growing evidence supporting the role of H_2S in stress mitigation, significant knowledge gaps remain regarding its precise mechanisms in salt stress contexts, optimal application strategies, and species-specific responses. Further, the role of H_2S

in promoting salt stress is still lacking in a large group of crops of agricultural and economic value. In addition, there remains a significant knowledge gap regarding the regulatory role of H_2S in nitrogen metabolism under salinity stress conditions, with current research on this specific interaction being notably limited in the scientific literature. Thus, exploring this area could yield valuable insights for fundamental plant physiology and practical agricultural applications for salt-affected soils, which constitute a growing global challenge and is surely going to enrich the discipline related to H_2S signaling. Hence, we hypothesize that H_2S plays a crucial regulatory role in mitigating NaCl-stress induced oxidative damage and nitrogen metabolism inhibition in sponge gourd and okra seedlings, potentially offering a protective mechanism that significantly ameliorates the adverse consequences of salinity stress. Novel approaches to strengthen plant resistance to salinity stress can be developed by examining how these plants respond to various stress treatments, including H_2S exposure. If effective, H_2S donors could offer relatively simple and cost-effective interventions for improving crop performance in saline environments, addressing food security concerns in regions where soil salinity limits agricultural productivity.

2.11. Objectives

To understand the mechanism of sodium chloride stress in some vegetable crops under the exogenous application of NaHS with or without H_2S scavenger- Hypotaurine, and H_2S inhibitor- DL-Propargylglycine, the following objectives are set forth:

- Analysis of biomass accumulation pattern and status of photosynthetic pigments by growing vegetable crop under NaCl stress with and without exogenous NaHS.
- Analysis of inorganic nitrogen contents and enzymes involved in nitrate and ammonia assimilation under NaCl stress with and without exogenous NaHS.
- Study of reactive oxygen species, oxidative damage to lipids and proteins, and the role of antioxidants in withstanding the stress.
- Understand the underlying signaling mechanism of H_2S in the test seedling during NaCl stress.

Chapter 3

3. METHODS AND MATERIALS

3.1.Test crops

Seeds were purchased from a certified supplier from Phagwara, Punjab, India, and were sterilized before their germination and growth. Sampling was done, and the herbarium was prepared when the plants attained the flowering stage. The herbarium was then sent for plant identification to Janaki Ammal Herbarium: Council of Scientific and Industrial Research-Indian Institute of Integrative Medicine at (CSIR-IIIM), Jammu. The *Luffa aegyptiaca* Mill (sponge gourd) and *Abelmoschus esculentus* (L.) okra plants were identified under accession no. RRLH:30033 and RRLH:30032, respectively, by a renowned taxonomist, Dr. Manu Khajuria (Annexure 1). The whole study was conducted in the Department of Botany, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India.

3.2.Growth conditions of sponge gourd and okra seedlings

Healthy seeds of sponge gourd and okra were taken based on their uniformity. Seeds were surface sterilized with sodium hypochlorite (2%; v/v) for 15 minutes, followed by thorough rinsing with double-distilled water and a 2-3-hour incubation in double-distilled water. They were then allowed to germinate in the dark in petri plates lined with muslin cloth at 25±2°C for two days. Uniformly germinated seeds were transferred to plastic trays containing sterilized acid-washed sand and maintained under controlled environmental conditions: temperature of 25±2 °C, photon density of 250 µmol photons $m^{-2}s^{-1}$, and a 16:8-hour (day: night) photoperiod for 10 days. Daily spraying with sterilized double-distilled water was performed at 10 a.m. until the complete development of primary leaves. Subsequently, the seedlings were carefully plucked, thoroughly cleaned with double-distilled water, and acclimated to Hoagland media for 24 hours before initiating hydroponic treatments.

3.3.Chemicals, glassware, and instruments

All glassware, including beakers, flasks, petri dishes, pipettes, and test tubes utilized in the current investigation, was manufactured by Borosil. The chemicals and solvents utilized were of analytical grade and procured from manufacturers such as Sigma Aldrich, Loba, Himedia, Merck, and CDH. All reagents and standards were produced

in double-distilled water to ensure consistency and reproducibility of results. The instruments used in the present study are listed in the Annexure.

3.4. Nutrient media

The nutritional medium of Hoagland and Arnon at half strength (Hoagland and Arnon, 1938) was added to the seedlings. It contained macronutrients (g L^{-1}) and micronutrients (mg L^{-1}) (Table 3.1).

Table 3.1. Represents the nutritional media composition.

Macronutrients	
Nutrient	**Weight (g L^{-1})**
Potassium dihydrogen sulfate (KH_2PO_4)	0.034
Magnesium sulfate ($MgSO_4.7H_2O$)	0.123
Potassium nitrate (KNO_3)	0.126
Calcium nitrate ($Ca(NO_3)_2$)	0.271
Micronutrients	**(mg L^{-1})**
Sodium molybdate (Na_2MoO_4)	0.045
Copper sulfate ($CuSO_4.5H_2O$)	0.045
Zinc sulfate ($ZnSO_4.7H_2O$)	0.110
Manganese chloride ($MnCl_2.4H_2O$)	0.910
Boric acid (H_3BO_3)	1.430

Ferric chloride ($FeCl_3$)	9.680
Ethylenediaminetetraacetic acid (EDTA)	30.00

3.5.Sterilization process of growing media and glassware

The sand used for the plantation of seeds undergoes a stringent sterilization process. It has been thoroughly rinsed with tap water, and subsequently treated with concentrated hydrochloric acid (HCl) for 24 hours to eliminate all organic residue. The sand was meticulously rinsed with tap water, followed by double-distilled water after 24 hours. Lastly, the dried acid-washed sand was placed for two hours at 250 °C in a hot air oven. The sterilizing of glassware and Hoagland nutrient media was conducted using an autoclave (Model no. CC-3126, Arsh Enterprise, India) at a pressure and temperature of 15 *lbs* inch^{-2}, and 121 °C respectively for 15 minutes. Whereas, the plastic cups and trays utilized in this investigation were rinsed and surface sterilized with absolute alcohol (100%).

3.6.Preparation and mode of NaCl, NaHS, PAG, and HT treatment

Sodium chloride (chemical formula= NaCl; molecular mass= 58.44 g mol^{-1}; IUPAC name: sodium chloride; purity = 99.5%; CAS No. 7647-14-5; Merck), Sodium hydrogen sulfide (chemical formula= NaHS; molecular mass= 56.063g mol^{-1}; IUPAC name: sodium hydrosulfide; purity = 30% w/v extra pure; CAS No. 207683-19-0; Merck) are used for salt and H_2S treatment.

Hypotaurine (chemical formula= $C_2H_7NO_2S$; molecular mass= 109.15 g mol^{-1}; IUPAC name: 2-Aminoethanesulfinic acid; purity= ≥98%; CAS No. 300-84-5; Merck) and DL-DL-Propargylglycine (chemical formula= $C_5H_7NO_2$; molecular mass= 113.11g mol^{-1}; IUPAC name; 2-Amino-4-pentynoic acid; purity= ≥98%; CAS No. 64165-64-6; Merck) were used as scavenger and inhibitors of H_2S.

To prepare a stock solution of 500 mM NaCl, 2.92 g of NaCl was dissolved in 100 mL of double-distilled water. Similarly, for NaHS (30% W/V), 1.87 mL of NaHS was added to 98.*13 mL* of double-distilled water to make 100 mM stock solution. This stock

solution was diluted via two steps to make a final stock solution of 1000 µM. Now this solution is further diluted to working solutions of different NaHS concentrations. Similarly, for PAG, a stock solution of 1000 µM was made by dissolving 5.6555 mg of PAG to 50 mL of double-distilled water. A stock solution of HT was made similarly by dissolving 10.915 mg of HT into 100 mL of double-distilled water. The PAG and HT at concentrations of 100 µM and 200 µM were incorporated into the Hoagland media containing seedlings cultivated at previously described conditions to check the effect of the inhibitor and scavenger of H_2S. The seedlings were taken after seven days of treatment, and many characteristics, including shoot and root fresh mass and lengths, were evaluated.

3.7.Treatment design of experiment

The experiment was conducted by exposing the seedlings to a different set of treatments, as presented in figure 3.1.

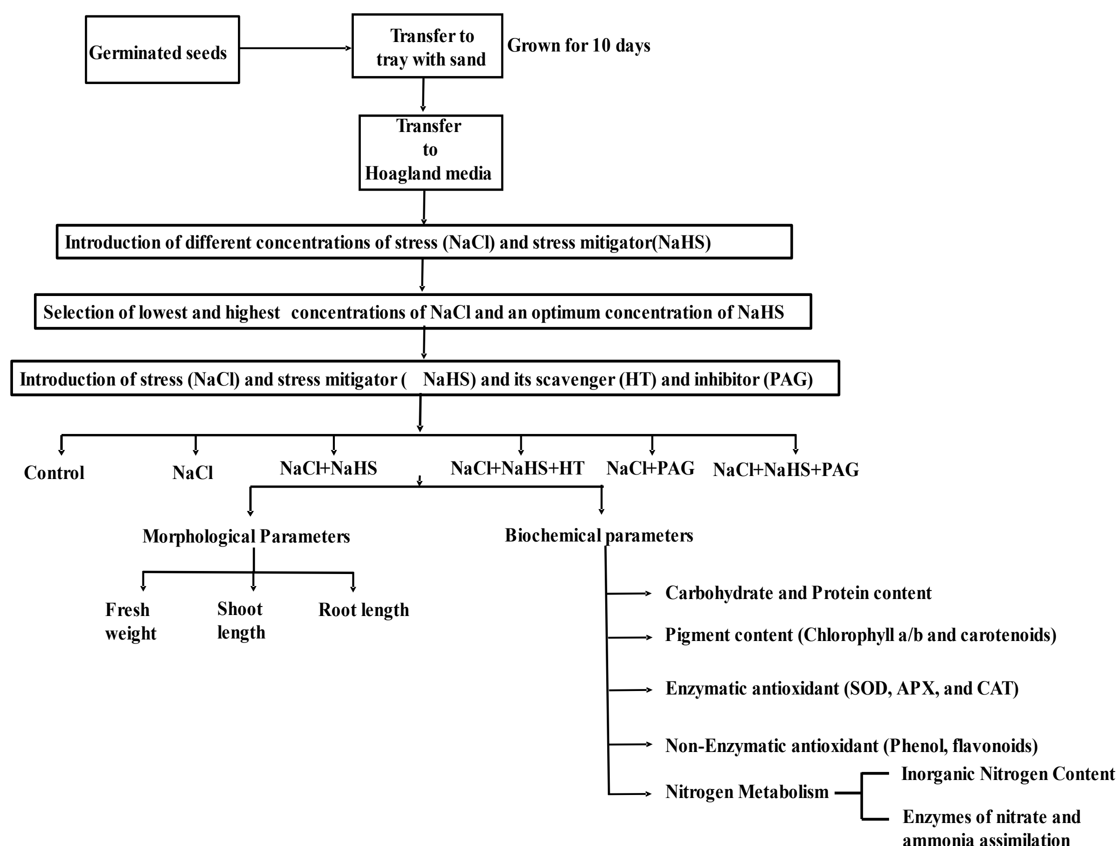

Figure 3.1. The figure provides a schematic representation of the experimental layout employed in the present study.

3.8.Evaluation of morphological and biochemical attributes

3.8.1. Estimation of morphological characteristics

Morphological attributes were evaluated based on the fresh weight, shoot, and root length measurements. A single pan electronic balance (Model-PGB220, Wensar, India) was used to record the fresh weight (FW) after seedlings were blotted and the length of the shoot and root was measured using a meter scale. The percentage change in seedlings was calculated using the following equation:

Change in FW (%) = $[(FW_{after} - FW_{before})/FW_{before}]*100$

3.8.2. Quantification of photosynthetic pigments

Chlorophyll is a key light-absorbing pigment with a magnesium-centered porphyrin ring structure. It exists in two main forms in plants - Chlorophyll a and b, which differ only in one side chain ($-CH_3$ in a versus -CHO in b). These pigments can be easily extracted using acetone and have absorption peaks at 663 nm and 646 nm, respectively. Similarly, carotenoids can be readily extracted using acetone, having absorption maxima at 470 nm.

Reagents

Acetone	80%

Procedure

To determine the pigment content (chlorophylls and carotenoids), fresh leaf (0.02 g) was taken from the differently treated test seedlings and was macerated in acetone (80%), followed by centrifugation at 4°C (13,000 rpm) for 20 minutes. Finally, it was quantified using a UV-visible spectrophotometer (Shimadzu Corp. model no. 80485) at 663, 646, and 470 nm (Sharma et al., 2022).

The following equations are used to quantify the pigments (mg/g FW);

Chlorophyll a= $12.25(A_{663}) – 2.79 (A_{646}) * (V/ 1000 * W)$……………(1)

Chlorophyll b= $21.50(A_{646})–5.10(A_{663}) * (V/ 1000 * W)$………………(2)

Total carotenoids= $[(1000* A_{470} – 1.82(Chla) – 85.02(Chlb)]/ 198* (V/ 1000 * W)$………(3)

V: The volume of acetone and W: Weight of the sample.

3.8.3. Assessment of carbohydrate content

The Anthrone test is used for the assessment of carbohydrates. This method is based on the reaction of carbohydrates with concentrated sulfuric acid to yield hydroxymethyl furfural. The resulting furfural combines with anthrone to form a compound with a blue-green hue.

Reagents

Methanol	100%
Anthrone- concentrated sulfuric acid	10 mM

Procedure

A fresh plant sample (0.1g) was taken from the differently treated seedlings and was macerated in methanol (Sharma et al., 2022). The supernatant (0.1 mL) was then put in a reaction mixture containing anthrone and distilled water, followed by centrifugation (10,000rpm; 10minutes) and quantification at 620 nm using a UV-visible spectrophotometer. A standard curve with a known glucose concentration was employed to determine carbohydrate content in the test seedlings.

3.8.4. Assessment of protein content

The Bradford assay is used for the assessment of proteins. The method is based on the binding of proteins with Coomassie Brilliant Blue dye. Their binding causes a transition in dye absorption spectra from 465nm to 595 nm, resulting in a color change from brown to blue.

Reagents

Protein buffer	Acetone	100 mL
	Trichloroacetic acid (TCA)	10 %
	β-mercaptoethanol (β-ME)	7 µL
Bradford	Ethanol	25 mL
	Orthophosphoric acid	50 mL
	Coomassie brilliant blue G-250	50 mg
	Double-distilled water	425 mL

Procedure

The 0.1 g fresh plant sample was extracted from the differently-treated test seedlings and was macerated in the protein extraction buffer (Sharma et al., 2020). Protein extraction buffer was prepared using TCA (10%), β-ME (7 μL), and 100% acetone. Subsequently, using the Bradford reagent, the extracted sample was examined for the assessment of protein at 595 nm by UV-visible spectrophotometer.

3.8.5. Determination of nitrogen metabolism

3.8.5.1.Estimation of inorganic nitrogen content

3.8.5.1.1. Determination of nitrate content

This method is based on the reaction of nitrate with salicylic acid under an acidic environment to yield nitrosalicylic acid. This complex turns yellow in the basic environment (pH > 12) and displays an absorbance peak at 410 nm.

Reagents

Potassium phosphate buffer (K-P buffer)	0.1M (pH=7)
Salicylic acid- concentrated sulfuric solution	5%
Sodium hydroxide	2 N

Procedure

The plant sample (0.1g) was extracted from the differently treated fresh seedlings and was macerated in the K-P buffer (0.1M; pH 7). Subsequently, the supernatant (0.2 mL) was then put in a reaction blend encompassing 800 μL of salicylic acid-sulfuric acid solution (5% w/v), and 19 mL of sodium hydroxide (2 N), followed by an absorption estimation at 410 nm using a UV-visible spectrophotometer (Cataldo et al., 1975). To determine nitrate content in the test seedlings, a standard curve of potassium nitrate was used.

3.8.5.1.2. Determination of nitrite content

This method is based on the reaction of nitrite with sulphanilamide under a highly acidic environment to yield a diazonium compound. This compound produces an azo dye through diazo-coupling with N-1-Naphthylethylene diamine dihydrochloride (NEDD) that exhibits an absorbance peak at 540 nm.

Reagents

<table>
<tr><td rowspan="4">HEPES-buffer (pH=7.6)</td><td>HEPES</td><td>0.05 M</td></tr>
<tr><td>Potassium hydroxide</td><td>1 N</td></tr>
<tr><td>EDTA disodium salt</td><td>0.001 M</td></tr>
<tr><td>Cysteine</td><td>0.007 M</td></tr>
<tr><td colspan="2">Sulfanilamide-HCl</td><td>1%-1 N</td></tr>
<tr><td colspan="2">NEDD</td><td>0.02%</td></tr>
</table>

Procedure

The plant sample (0.1 g) was extracted from the variously treated seedlings and was macerated in extraction buffer consisting of 0.05 M HEPES maintained at pH 7.6 with 1N potassium hydroxide and 0.001 M EDTA, and 0.007 M cysteine. Subsequently, the supernatant was put in a 3 mL reaction blend encompassing 1 mL of sulphanilamide (1% w/v in 1 N HCl), 1 mL NEDD, and double-distilled water, followed by an absorption estimation at a wavelength of 540 nm (Hachiya, and Okamoto, 2017). To quantify the nitrite content in the test samples, a standard reference curve of potassium nitrite was used.

3.8.5.1.3. Determination of ammonia content

The solution of the Nessler reagent contains two components: potassium hydroxide and potassium mercuric iodide. Iodide and mercury ions combine with ammonia in an alkaline environment to generate a complex that is yellowish brown in hue and absorbs strongly at 420 nm.

Reagents

K-P buffer	0.1 M (pH=7)
Sodium potassium tartrate	10%
Nessler reagent	Commercial supply

Procedure

The 0.1 g of differently treated seedlings was extracted in the K-P buffer (0.1M; pH 7). Subsequently, the supernatant was placed in a reaction encompassing 20µL sodium

potassium tartrate (10%), double-distilled water, and 200 µL Nessler reagent, followed by an absorption estimation at 425 nm using a UV-visible spectrophotometer (Molins-Legua et al., 2006). To determine ammonia content in the test seedlings, a standard curve of ammonium chloride was used.

3.8.5.2.Nitrate assimilation enzymes

3.8.5.2.1. Quantification of nitrate reductase activity

The primary enzyme in nitrogen metabolism, nitrate reductase, converts nitrate into nitrite in a manner that is dependent on NAD(P)H. As a result, its activity may be measured by measuring the absorbance of its final product, nitrite, at 540 nm.

Reagents

<table>
<tr><td rowspan="4">Extraction Buffer</td><td>K-P buffer</td><td>0.1 M (pH=7.4)</td></tr>
<tr><td>Cysteine</td><td>0.0075 M</td></tr>
<tr><td>EDTA</td><td>0.001 M</td></tr>
<tr><td>Casein</td><td>1.5%</td></tr>
<tr><td colspan="2">K-P buffer</td><td>0.1 M (pH=7.5)</td></tr>
<tr><td colspan="2">Potassium nitrate</td><td>0.1 M</td></tr>
<tr><td colspan="2">Nicotinamide adenine dinucleotide (NADH)</td><td>2 mM</td></tr>
<tr><td colspan="2">Sulphanilamide -HCl</td><td>1%-1N</td></tr>
<tr><td colspan="2">NEDD</td><td>0.02%</td></tr>
</table>

Procedure

The plant sample (0.1 g) was extracted from the differently treated test seedlings (fresh) and was macerated in the K-P buffer (0.1 M; pH 7.4) containing cysteine (0.0075 M), EDTA (0.001 M), and casein (1.5%; pH 7.4). Subsequently, the 200 µL supernatant was then used in a reaction blend comprising 0.5 mL K-P buffer (0.1M; pH 7.5), 0.2 mL potassium nitrate, 0.4 mL NADH, and double-distilled water to start the reaction, followed by an hour incubation. The inclusion of sulphanilamide (1%): HCl (1N) solution and NEDD (0.02%) then terminates the reaction. The reaction mixture is then subjected to the measurement of absorption using a UV-visible spectrophotometer at

540 nm wavelength (Mengel et al., 1983). To determine nitrate reductase activity in the test seedlings, a standard curve of sodium nitrite was used.

3.8.5.2.2. Quantification of nitrite reductase activity

Nitrite reductase converts nitrite into ammonia, and thus the decrease in the amount of nitrite in the reaction mixture serves as the basis for the nitrite reductase activity assay.

Reagents

Extraction Buffer	K-P buffer	0.1 M (pH= 7.4)
	Cysteine	0.0075 M
	EDTA	0.001 M
	Casein	1.5%
K-P buffer		0.1 M (pH= 7.4)
Sodium nitrite		15 mM
Methyl viologen		5 mM
Sodium dithionite		86.15 mM
Sodium bicarbonate		190 mM
Sulphanilamide -HCl		1%-1N
NEDD		0.02%

Procedure

The plant sample (0.1 g) was extracted from the differently treated test seedlings (fresh) in a similar manner to that of nitrate reductase. Subsequently, the 100 µL supernatant was then used in a reaction blend comprising 0.4 mL K-P buffer (0.1 M; pH 7.4), 0.1mL sodium nitrite (15 mM), 0.2 mL methyl viologen (5 mM), 0.2mL sodium dithionite (86.15 mM) in a 190 mM $NaHCO_3$. A vigorous agitation on the vortex terminated the reaction. Following diazotation with 1 mL of sulfanilamide (1%): HCl (1 N), and 1 mL of NEDD (0.02%), nitrite ions were measured by subjecting the reaction mixture to the measurement of absorption using a UV-visible spectrophotometer at 540 nm wavelength (Debouba et al., 2006). To determine nitrite reductase activity in the test seedlings, a standard curve of sodium nitrite was used.

3.8.5.3.Ammonia assimilation enzymes (GS-GOGAT pathway)

3.8.5.3.1. Determination of the activity of glutamine synthetase

The enzymatic activity of glutamine synthetase is ascertained by measuring γ-glutamyl hydroxamate, which is produced from glutamate and hydroxylamine, through the γ-

glutamyl transferase reaction. When γ-glutamyl hydroxamate reacts with acidified ferric chloride, it produces a brown color that may be measured spectrophotometrically at 540 nm, allowing for quick quantification of glutamine synthetase activity.

Reagents

Extraction Buffer	Tris-HCl	50 mM(pH=7.8)
	Glycerol	15%
	2-mercaptoethanol	14 mM
	EDTA	1 mM
	Triton X-100	0.1%
L-glutamate		50 mM
Adenosine triphosphate (ATP)		10 mM
$MgSO_4$		30 mM
Hydroxylamine		20 mM
Tris-HCl		100 mM
Iron chloride		2.5%
TCA-HCl		(5%-1.5 N)

Procedure

The plant sample (0.1 g) was extracted from the differently treated test seedlings (fresh) and was macerated in Tris-HCl (50 mM; pH 7.8) containing glycerol (15%), 2-mercaptoethanol (14 mM), EDTA (1 mM), and triton X-100 (0.1%). Subsequently, 50 μL of the supernatant was mixed in a total reaction blend of 1 mL (pH of 8) comprising L-glutamate (50 mM), ATP (10 mM), $MgSO_4$ (30 mM), hydroxylamine (20 mM), and tris-HCl (100 mM). The reaction mixture was incubated at 27 °C and finally terminated with 2 mL of iron chloride (2.5%) and TCA-HCl (5%-1.5 N). Finally, the absorbance of the reaction mixture was monitored to evaluate glutamine synthetase activity at 540 nm by using a standard curve of L-Glutamic acid γ-mono-hydroxamate (Lillo, 1984).

3.8.5.3.2. Determination of the activity of glutamate synthase

To measure glutamate synthase, the rate at which NADPH or NADH oxidizes is estimated by measuring the change in absorbance at 340 nm after the enzyme extract is added.

Reagents

<table>
<tr><td rowspan="5">Extraction Buffer</td><td>Sodium phosphate buffer (Na-P buffer)</td><td>0.2 M (pH=7.5)</td></tr>
<tr><td>EDTA</td><td>2 mM</td></tr>
<tr><td>Potassium chloride (KCl)</td><td>50 mM</td></tr>
<tr><td>β-ME</td><td>0.1%</td></tr>
<tr><td>Triton X-100</td><td>0.5%</td></tr>
<tr><td colspan="2">Na-P buffer and EDTA</td><td>25 mM (pH=7.5) and 1 mM respectively</td></tr>
<tr><td colspan="2">L-glutamine</td><td>20 mM</td></tr>
<tr><td colspan="2">2-oxoglutarate</td><td>5 mM</td></tr>
<tr><td colspan="2">KCl</td><td>100 mM</td></tr>
<tr><td colspan="2">NADH</td><td>1 mM</td></tr>
</table>

Procedure

The plant sample (0.1 g) was extracted from the differently treated test seedlings (fresh) and was macerated in Na-P buffer (0.2 M; pH:7.5) containing EDTA (0.002 M), potassium chloride (KCl) (0.05 M), β-ME (0.1%) and Triton X-100 (0.5%). Subsequently, 500 µL of the supernatant was mixed in a reaction blend comprising 0.4 mL, L- glutamine (0.02 M), 0.4 mL, 2-oxoglutarate (0.005 M), 0.1 mL KCl (0.1 M), 0.6ml NADH (0.001 M) and Na-P buffer (0.025 M) encompassing EDTA; pH 7.5. At last, for five minutes, the reaction mixture's absorbance was monitored to evaluate glutamate synthase activity at 340nm (Singh and Srivastava, 1986).

3.8.5.3.3. Determination of glutamate dehydrogenase activity

Glutamate dehydrogenase is determined by monitoring the reduced NADH oxidation at 340 nm.

Reagents

Extraction Buffer (pH=7.4)	Na-P buffer	0.05 M
	Sucrose	0.4 M
	EDTA disodium salt	2 mM
	Cysteine	1 mM
Na-P buffer		0.1 M (pH=8.1)
2-oxoglutarate		0.2 M
Ammonium sulfate		1.5 M
NADH		1 mM

Procedure

The plant sample (0.1 g) was extracted from the differently treated test seedlings(fresh) and was macerated in Na-P buffer (0.05 M; pH:7.4) encompassing sucrose (0.4 M), EDTA disodium salt (2 mM), and cysteine (1 mM; pH:7.4). Subsequently, the 100 µL supernatant was then used in a reaction blend comprising 1.7 mL of Na-P buffer (0.1 M; pH:8.1), 0.3 mL of 2-oxoglutarate(2-OG) (0.2 M), 0.3 mL ammonium sulfate (1.5 M), and 0.6 mL NADH (1 mM) to initiate the reaction. Finally, glutamate dehydrogenase activity was assessed by tracking the absorbance change at 340 nm for five minutes (Singh and Srivastava, 1983).

3.8.6. Measurement of oxidative stress

3.8.6.1.Estimation of hydrogen peroxide content

A potassium iodide-based approach is used to quantify hydrogen peroxide content. In an acidic medium, hydrogen peroxide oxidizes the iodide ions of potassium iodide to iodine. After reacting with iodide, iodine ions form triiodide, or a yellow solution, which can be measured using spectrophotometry at 390 nm.

Reagents

TCA	0.1%
K-P buffer	0.1 M (pH=7)
Potassium iodide	1 M

Procedure

The plant sample (0.1 g) was taken from the differently-treated fresh seedlings and homogenized in TCA (0.1%) (Gautam et al., 2023). A UV-visible spectrophotometer was used to quantify the supernatant's concentration at 390 nm after it had been added to a reaction mixture that contained 0.5 mL of K-P buffer (0.1 M) and 1 mL of potassium iodide (1 M). To quantify hydrogen peroxide content in the test samples, a hydrogen peroxide standard reference curve was employed.

3.8.6.2.Estimation of lipid peroxidation via Malondialdehyde content

Malondialdehyde is created when polyunsaturated fatty acids undergo enzymatic degradation and auto-oxidation. A spectrophotometric measurement of the orange-pinkish hue produced by the combination of malondialdehyde and 2-thiobarbituric acid (TBA) in an acidic environment through a nucleophilic addition reaction with absorbance maxima at 532 nm is used to evaluate the degree of lipid peroxidation.

Reagents

TCA	0.1%
TBA and TCA solution	0.5% and 20%, respectively

Procedure

The plant sample (0.1 g) was extracted from the differently treated test seedlings and was macerated in 0.1% TCA (Guleria and Yadav, 2014). The supernatant (0.5 mL) was then put in a 2 mL reaction mixture encompassing 0.5% TBA in 20% TCA, followed by its quantification at two different wavelengths, 532 and 600 nm, using a UV-visible spectrophotometer. A 155 mM^{-1} cm^{-1} extinction coefficient was employed to analyze the content of malondialdehyde in the test sample.

3.8.7. Assessment of enzymatic and non-enzymatic antioxidants

3.8.7.1. Enzymatic antioxidants

3.8.7.1.1. Determination of superoxide dismutase activity

The activity of the superoxide dismutase enzyme is measured by the suppression of nitroblue tetrazolium dye photoreduction by superoxide radicals in the presence of EDTA, which causes a rise in absorbance at 560 nm as a result of formazon production.

Reagents

K-P buffer	100 mM (pH=7); 50 mM (pH=7)
EDTA	0.1 µM
Methionine	13 µM
Nitroblue tetrazolium	75 µM
Riboflavin	2 µM

Procedure

The plant sample (0.1 g) was extracted from the differently treated test seedlings and was homogenized in K-P buffer (0.1 M; pH 7). Subsequently, the supernatant (100 µL) was placed in a reaction mixture encompassing 280 µL each of K-P buffer (0.05 M), EDTA (0.1µM), methionine (13 µM), nitroblue tetrazolium (75 µM), and riboflavin (2 µM) for the assessment of superoxide dismutase activity at 560 nm using a UV-visible spectrophotometer (Gautam et al., 2023).

3.8.7.1.2. Determination of ascorbate peroxidase activity

Ascorbate peroxidase activity was estimated by recording the ascorbate oxidation at 390 nm. Ascorbate peroxidase produces mono-dehydroascorbic acid by reducing hydrogen peroxide to water while employing ascorbic acid as a substrate.

Reagents

K-P buffer	50 mM (pH=7)
Hydrogen peroxide	25 µM
Ascorbic acid	0.0005 M
EDTA	0.2 µM

Procedure

The plant sample (0.1 g) was extracted from the differently treated test seedlings and was macerated in 2 mL K-P buffer (50 mM; pH 7). Subsequently, the supernatant (200 µL) was then put in a reaction mixture encompassing 500 µL each of K-P buffer (50 mM), hydrogen peroxide (25 µM), ascorbic acid (0.0005 M), and EDTA (0.2 µM), followed by the quantification of enzyme activity for 1 minute at 290nm using a UV-visible spectrophotometer (Gautam et al., 2023).

3.8.7.1.3. Determination of catalase activity

The activity of catalase is determined by the titrimetric oxidation of hydrogen peroxide with Potassium permanganate ($KMnO_4$) to analyze the amount of residual hydrogen peroxide in the reaction mixture.

Reagents

Reagent	Concentration
Na-P buffer	100 mM (pH=6.8)
K-P buffer	100 mM (pH=7)
EDTA	0.1 µM
Hydrogen peroxide	20 mM
Sulfuric acid	2%
$KMnO_4$	0.01 N

Procedure

The plant sample (0.1 g) was extracted from variously treated test seedlings and was macerated in 2 mL of Na-P buffer (0.1M; pH 6.8). Subsequently, the catalase activity was accessed through titration with $KMnO_4$ (0.01N) by examining the supernatant (1 mL) in a reaction mixture encompassing distilled water (3.98 mL), 4µL EDTA (0.1 µM), 4µL hydrogen peroxide (0.02 M), 12 µL K- P buffer (0.1 M; pH 7), and 10 mL of 2% sulfuric acid till the appearance of a tint of pink (Gautam et al., 2023).

3.8.7.2. Nonenzymatic antioxidants

3.8.7.2.1. Assessment of total phenolic content

Under an alkaline environment, phenol reacts with phosphomolybdic acid in the folin-ciocalteau reagent to form a blue-colored complex known as molybdenum blue, which is detectable at 725 nm.

Reagents

Methanol	80%
Folin-ciocalteu reagent	Commercial supply
Sodium carbonate	20%

Procedure

The plant sample (0.1g) was taken from the differently-treated test seedlings and was macerated in 80% methanol (Sharma et al., 2022). Subsequently, the folin-ciocalteu reagent, distilled water, and 20% sodium carbonate were used as a reaction mixture to record the absorbance in the test sample at 725nm using a UV-visible spectrophotometer. Lastly, to quantify the total phenolic content in the test seedlings, a standard reference curve of gallic acid was used.

3.8.7.2.2. Assessment of flavonoid content

The idea underlying the colorimetric approach based on aluminum chloride for flavonoids detection is that $AlCl_3$ combines with the C-4 keto groups and either the C-3 or C-5 hydroxyl groups of flavones and flavonols to produce acid-stable complexes. Furthermore, it develops acid-labile complexes with the ortho dihydroxyl groups found in flavonoids' A- or B-rings.

Reagents

Methanol	80%
Aluminum chloride	10%
Potassium acetate	1M

Procedure

The plant sample (0.1g) was extracted from the differently-treated test seedlings (fresh) and was macerated in 80% methanol (Gautam et al., 2020). Subsequently, the supernatant (1 mL) was then put in a 2 mL reaction mixture encompassing aluminum chloride (200µL), potassium acetate (200 µL), and distilled water (600 µL), followed by its quantification at 415nm using a UV-visible spectrophotometer. Lastly, to quantify the flavonoids, a standard reference curve of rutin was used.

3.8.8. Assessment of antioxidant potential (DPPH assay)

The DPPH assay operates on the principle that antioxidants neutralize/scavenge DPPH, resulting in the decolorization of the purple DPPH solution. The color of the DPPH solution will decrease more when the antioxidant's capacity to scavenge free radicals increases.

Reagents

Methanol	100%
DPPH	84.5 µM

Procedure

The antioxidant potential was assessed by performing the DPPH assay (Guleria and Yadav 2014). The plant sample (0.1 g) was extracted from the differently treated test seedlings and was then macerated in methanol (100%), followed by quantification using a UV-visible spectrophotometer at 517 nm against methanolic-DPPH solution.

3.8.9. Determination of H_2S content

This approach quantifies H_2S content as equivalents of 5-thio-2-nitrobenzoate anions. These anions are produced through the interaction of H_2S with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which displays an absorbance maximum of 412 nm.

Reagents

K-P buffer containing EDTA (10 mM)	100 mM (pH=7)
DTNB	20 mM

Procedure

The plant sample (0.1g) was extracted from the differently treated test seedlings (fresh) and was macerated in the K-P buffer (100 mM; pH 7) comprising EDTA (10 mM). Subsequently, the supernatant (0.1 mL) was put in a 2 mL reaction mixture encompassing extraction buffer and 20 µL Ellman's reagent (20 mM), followed by the estimation of absorption at 412 nm using a UV-visible spectrophotometer (Mostofa et al., 2015a). To determine H_2S content in the test seedlings, a standard curve of NaHS was used.

3.8.10. Statistical analysis

Three separate replicates were used in this study. Data presented in figures and tables indicate the mean ± standard deviation of three biological replicates. Statistical evaluation was performed using analysis of variance, with mean values compared using Duncan's Multiple Range Test.

Chapter 4

4. RESULTS AND DISCUSSION

Enhanced salt in agricultural landholdings has turned out to be a universal environmental issue, seriously affecting crop productivity (Zorb et al., 2019). Toxic levels of NaCl trigger reactive oxygen species (ROS) production, which breaks down cellular biomolecules and causes the disintegration of biomembrane, thereby hampering the general biological processes of plants (Li et al., 2020a; Karle et al., 2021; Wei et al., 2021b). H_2S has been identified as a potential endogenous signaling molecule regulating various biological processes in plants, most importantly the alleviation of stress tolerance under harsh environmental circumstances (Corpas et al., 2019; Kaur et al., 2022). The role of H_2S in regulating plant responses during salt stress has been reported for some plants, including *S. italica* L. and *Arabidopsis thaliana*, but with limited exploration of the role of nitrogen metabolism (Mostofa et al., 2015a; Ding et al., 2019; Wei et al., 2021b; Yang et al., 2023; Zhang et al., 2023b). Moreover, no such study has been reported for the role of H_2S in regulating NaCl stress in sponge gourd and okra. Hence, the present study documents the potential of H_2S for salt stress regulation of sponge gourd and okra, with a focus on understanding the importance of nitrogen metabolism for stress tolerance.

4.1. Determination of appropriate salt concentrations to be used for the sponge gourd study

The sponge gourd seeds were germinated and then hydroponically exposed to salt concentrations, 25 mM (S1), 50 mM (S2), and 75 mM (S3) for seven days. The shoot length was reduced by 22, 40, and 60% in S1, S2, and S3 treated sponge gourd compared to the control after hydroponic treatment, respectively (Table 4.1; Fig. 4.1). It indicated that an enhancement in salt concentration inhibited the shoot elongation in the treated seedlings. Likewise, the root length was reduced by 27, 39, and 58% in S1-S3 exposed sponge gourd, compared to control plants (Table 4; Fig. 4.1). Similarly, a change in salt concentration from 25-75 mM reduced the fresh weight accumulation of sponge gourd by 18, 34, and 46% compared to the control (Table 4.1).

Figure 4.1. The figure depicts the effect of NaCl on the morphological parameters of sponge gourd seedlings.

Further, NaCl exposure significantly reduced the tested biochemical parameters, chlorophyll a, chlorophyll b, carotenoids, carbohydrates, and protein content in salt-stressed sponge gourd than the control. The antioxidant potential, enzymatic and non-enzymatic antioxidant parameters of salt-stressed sponge gourd were considerably enhanced compared to the control (Table 4.1). The total phenol content was enhanced by 17, 27, and 41% in S1, S2, and S3 seedlings, respectively, compared to the control. Similarly, the flavonoid content of S1, S2, and S3 sponge seedlings was enhanced by 11, 19, and 28% compared to the control. In salt-stressed sponge gourd, free radical scavenging potential was likewise elevated by 12, 25, and 34% in S1, S2, and S3 seedlings relative to the control. The enzymatic antioxidants exhibited a similar expression pattern; superoxide dismutase activity was upregulated in S1, S2, and S3 seedlings by 19, 37, and 55%, respectively, compared to the control. A similar pattern was exhibited by ascorbate peroxidase and it showed an upregulated expression on NaCl exposure in S1, S2, and S3 treated seedlings by 19, 43, and 63% compared to the control. Unlike the other antioxidant enzymes, catalase activity showed varied expression, it was enhanced by 25 and 42% in S1 and S2 treated seedlings, however, in S3, its activity was diminished by 16% compared to the control.

A simultaneous increase in the oxidative stress was observed in sponge gourd with enhancement in salt concentration from S1 to S3, as indicated by the hyperaccumulation of hydrogen peroxide and malondialdehyde levels compared to the control sponge gourd. The hydrogen peroxide content was elevated by 68, 122, and 169% in S1, S2,

and S3 sponge gourd seedlings than the control. The malondialdehyde content of S1, S2, and S3 treated sponge gourd seedlings exhibited a similar expression pattern and was increased by 43, 74, and 102% compared to the control. Hence, the lowest and highest, 25 mM (S1) and 75 mM (S3) NaCl concentrations were used for further experiments in the present study.

Table 4.1. Influence of varying NaCl concentrations on sponge gourd. The observed morphological and biochemical plant parameters were significantly reduced with an increase in NaCl concentration.

Parameters	C	S1	S2	S3
Shoot Length	16.55 ± 0.93^{a}	12.85 ± 0.98^{b}	9.87 ± 0.62^{c}	6.64 ± 0.71^{d}
Root Length	22.99 ± 0.66^{a}	16.76 ± 1.02^{b}	14.08 ± 0.71^{c}	9.66 ± 0.92^{d}
Fresh weight	27.37 ± 0.76^{a}	22.29 ± 0.98^{b}	18.13 ± 0.88^{c}	14.66 ± 0.93^{d}
Chlorophyll a	2.03 ± 0.05^{a}	1.87 ± 0.05^{b}	1.63 ± 0.04^{c}	1.36 ± 0.04^{d}
Chlorophyll b	0.76 ± 0.02^{a}	0.64 ± 0.03^{b}	0.54 ± 0.03^{c}	0.45 ± 0.04^{d}
Carotenoids	0.56 ± 0.003^{a}	0.52 ± 0.003^{b}	0.47 ± 0.003^{c}	0.41 ± 0.003^{d}
Carbohydrates	21.73 ± 0.67^{a}	19.59 ± 0.34^{b}	17.93 ± 0.34^{c}	15.22 ± 0.42^{d}
Protein	94 ± 2.27^{a}	83.82 ± 3.54^{b}	74.54 ± 3.03^{c}	65.25 ± 3.03^{d}
Hydrogen peroxide	0.52 ± 0.06^{d}	0.87 ± 0.079^{c}	1.15 ± 0.09^{b}	1.39 ± 0.09^{a}
Malondialdehyde Equivalents	20 ± 0.55^{d}	28.65 ± 0.73^{c}	34.84 ± 1.09^{b}	40.39 ± 1.28^{a}
Total phenol content	371.9 ± 8.62^{d}	434.25 ± 13.26^{c}	472.69 ± 7.95^{b}	523.31 ± 13.26^{a}
Flavonoids	0.35 ± 0.01^{d}	0.39 ± 0.01^{c}	0.42 ± 0.01^{b}	0.45 ± 0.004^{a}
Free radical scavenging potential	58.34 ± 1.34^{d}	65.46 ± 1.49^{c}	73.19 ± 1.89^{b}	78.42 ± 1.57^{a}
Superoxide dismutase	45.86 ± 2.93^{d}	54.59 ± 2.30^{c}	63.02 ± 1.67^{b}	71.15 ± 2.72^{a}
Ascorbate peroxidase	0.13 ± 0.01^{d}	0.16 ± 0.01^{c}	0.19 ± 0.01^{b}	0.22 ± 0.01^{a}
Catalase	86 ± 2.83^{c}	108 ± 5.66^{b}	122 ± 2.83^{a}	72 ± 5.66^{d}

★a, b, c, d indicate significant differences

4.2.Determination of NaHS concentration for the sponge gourd study

NaHS was used in the present study as a probable H_2S source to evaluate its potential for regulating the morphology and biochemical parameters in sponge gourd on salt stress exposure (Table 4.2). The sponge gourd seedlings were hydroponically exposed to 10 µM (N1), 25 µM (N2), 50 µM (N3) of NaHS. The shoot length elongation was enhanced by 31, 32, and 35% in sponge gourd treated with N1, N2, and N3, respectively however, the elongation was only 29% in untreated control seedlings (Table 4.2; Fig. 4.2). Likewise, in comparison to 16% increase in root length of control, the elongation was noticed to be 17-23% in sponge gourd exposed to N1-N3 NaHS, respectively (Table 4.2; Fig. 4.2). Likewise, the fresh weight was enhanced by33-38% in N1-N3 treated sponge gourd, in comparison to the 32% increase observed in the control (Table 4.2). Hence, the morphological parameters evaluation indicated N3 (50 µM) as the best NaHS concentration.

Figure 4.2. The figure depicts the potential of NaHS in improving sponge gourd morphology.

Similarly, the biochemical parameters were also evaluated in the presence of NaHS compared to the control. The accumulation of carbohydrates, proteins, and enzymatic as well as non-enzymatic antioxidant parameters was consistently increased with an increase in NaHS concentration from N1 to N3 concerning the control (Table 4.2). Simultaneously, the hydrogen peroxide and malondialdehyde levels were significantly reduced with NaHS exposure compared to the control (Table 4.2). The hydrogen peroxide content was reduced by 3, 8, and 17% in N1, N2, and N3 treated sponge gourd seedlings, respectively, compared to the control. The MDA accumulation of N1-N3 treated seedlings was likewise reduced by 4-32%, respectively, relative to the control.

Hence, the morphological and biochemical characterization of NaHS-treated sponge gourd indicated 50 µM (N3) as the significant concentration, and was therefore used in the rest of the experiments of the study.

Table 4.2. Influence of NaHS exposure on sponge gourd. The observed morphological and biochemical plant parameters were significantly enhanced with an increase in NaHS concentration.

Parameters	C	N1	N2	N3
Shoot Length	29.37 ± 0.72^{d}	30.78 ± 0.31^{c}	32.46 ± 0.70^{b}	35.16 ± 0.66^{a}
Root Length	16.34 ± 0.70^{d}	16.52 ± 0.69^{c}	18.98 ± 0.87^{b}	22.66 ± 0.47^{a}
Fresh weight	31.62 ± 0.92^{d}	33.21 ± 0.71^{c}	34.81 ± 0.49^{b}	38.25 ± 0.89^{a}
Chlorophyll a	1.91 ± 0.02^{c}	1.91 ± 0.03^{c}	1.98 ± 0.03^{b}	2.04 ± 0.02^{a}
Chlorophyll b	0.68 ± 0.02^{b}	0.69 ± 0.02^{b}	0.73 ± 0.02^{a}	0.76 ± 0.02^{a}
Carotenoids	0.48 ± 0.002^{d}	0.49 ± 0.002^{c}	0.51 ± 0.003^{b}	0.53 ± 0.002^{a}
Carbohydrates	17.82 ± 0.47^{c}	18.40 ± 0.44^{c}	19.41 ± 0.42^{b}	20.74 ± 0.39^{a}
Protein	96.5 ± 2.27^{c}	98.64 ± 2.78^{c}	107.04 ± 2.53^{b}	117.21 ± 3.28^{a}
Hydrogen peroxide	0.50 ± 0.03^{a}	0.48 ± 0.03^{a}	0.46 ± 0.02^{ab}	0.41 ± 0.02^{b}
Malondialdehyde Equivalents	22.96 ± 1.45^{a}	22.06 ± 1.28^{b}	19.74 ± 1.64^{b}	15.61 ± 1.27^{c}
Total phenol content	317.53 ± 11.26^{c}	327.37 ± 14.58^{bc}	340.96 ± 8.61^{b}	359.25 ± 13.25^{a}
Flavonoids	0.32 ± 0.01^{d}	0.35 ± 0.01^{c}	0.37 ± 0.01^{b}	0.39 ± 0.004^{a}
Free radical scavenging potential	61.67 ± 0.86^{d}	63.68 ± 1.17^{c}	66.52 ± 1.10^{b}	69.29 ± 0.94^{a}
Superoxide dismutase	43.19 ± 1.67^{c}	45.41 ± 1.04^{bc}	47.63 ± 1.25^{b}	50.14 ± 1.46^{a}
Ascorbate peroxidase	0.15 ± 0.01^{c}	0.16 ± 0.01^{b}	0.17 ± 0.01^{b}	0.18 ± 0.01^{a}
Catalase	84 ± 5.65^{c}	90 ± 2.82^{b}	94 ± 2.82^{b}	100 ± 5.65^{a}

★a, b, c, d indicate significant differences

Based on the above preliminary experiments, 25, 75 mM NaCl and 50 µM NaHS concentrations were identified as the appropriate concentrations. The sponge gourd was also exposed to H_2S inhibitor DL-propargylglycine (PAG) and H_2S scavenger

hypotaurine (HT) to validate the function of H_2S emitted from NaHS as an abiotic stress-tolerant molecule, as shown in Table 4.3. The 200 µM HT and 100 µM PAG was used during the study as per available literature (Iqbal et al., 2021; Mostofa et al., 2015b; Tang et al., 2020; Valivand et al., 2019; Janicka et al., 2017; Jia et al 2018; Husain et al., 2022; Husain et al., 2021; Ye et al., 2020).

Table 4.3. Experimental setup used in the present study to determine the potential of NaHS as an H_2S donor for inducing salt stress tolerance in sponge gourd.

C				
S1	S1N	S1NHT	S1PAG	S1NPAG
S3	S3N	S3NHT	S3PAG	S3NPAG

4.3. Morphological characterization of sponge gourd exposed to mixed treatments in hydroponics

The seedlings subjected to variable treatments of salt, NaHS, H_2S scavenger, and inhibitor designated as S1, S1N, S1NHT, S1PAG, S1NPAG, S3, S3N, S3NHT, S3PAG, and S3NPAG in the study were observed to show altered morphology as depicted in Fig. 4.3A. With the increase in NaCl concentration from S1 to S3, the seedlings exhibited 11 and 36% decrease in the shoot length change after treatment compared to the control, respectively (Fig. 4.3B). The root length measurement exhibited a similar trend, and was reduced by 17 and 45% in S1 and S3 seedlings, respectively, compared to the control (Fig. 4.3C).

NaCl-mediated hydroponic salt stress has been reported to considerably reduce the shoot and root length of plants including *S. italica* L., *A. thaliana*, *Fagopyrum tataricum*, *Triticum aestivum* L., *Oenanthe javanica*, *Phaseolus vulgaris* L., and *Vicia faba* (Deng et al., 2016; Kumar et al., 2021; Kumari et al., 2021; Mohamed et al., 2021; Dawood et al., 2022a, b; Yang et al., 2023; Zhang et al., 2023b; Zhang et al., 2023c). Recently, PLETHORA1/2 (PLT1/2) transcription factor-mediated regulation of root meristem during salt stress has been reported. The study has thus documented that salt stress-mediated reduction of meristematic activity was responsible for decreased root length (Hao et al., 2023). So, the reduced root meristematic growth of salt stress-treated

sponge gourd might be responsible for their reduced root length in the present study. Further, the plant root length is directly related to its nutrient uptake and thus provides sufficient nutrient supply to the above-ground plant parts and proper plant growth (Gautam et al., 2023). Thus, in the present study, the reduced root length of salt-stressed sponge inhibited the nutrient supply and decreased shoot elongation.

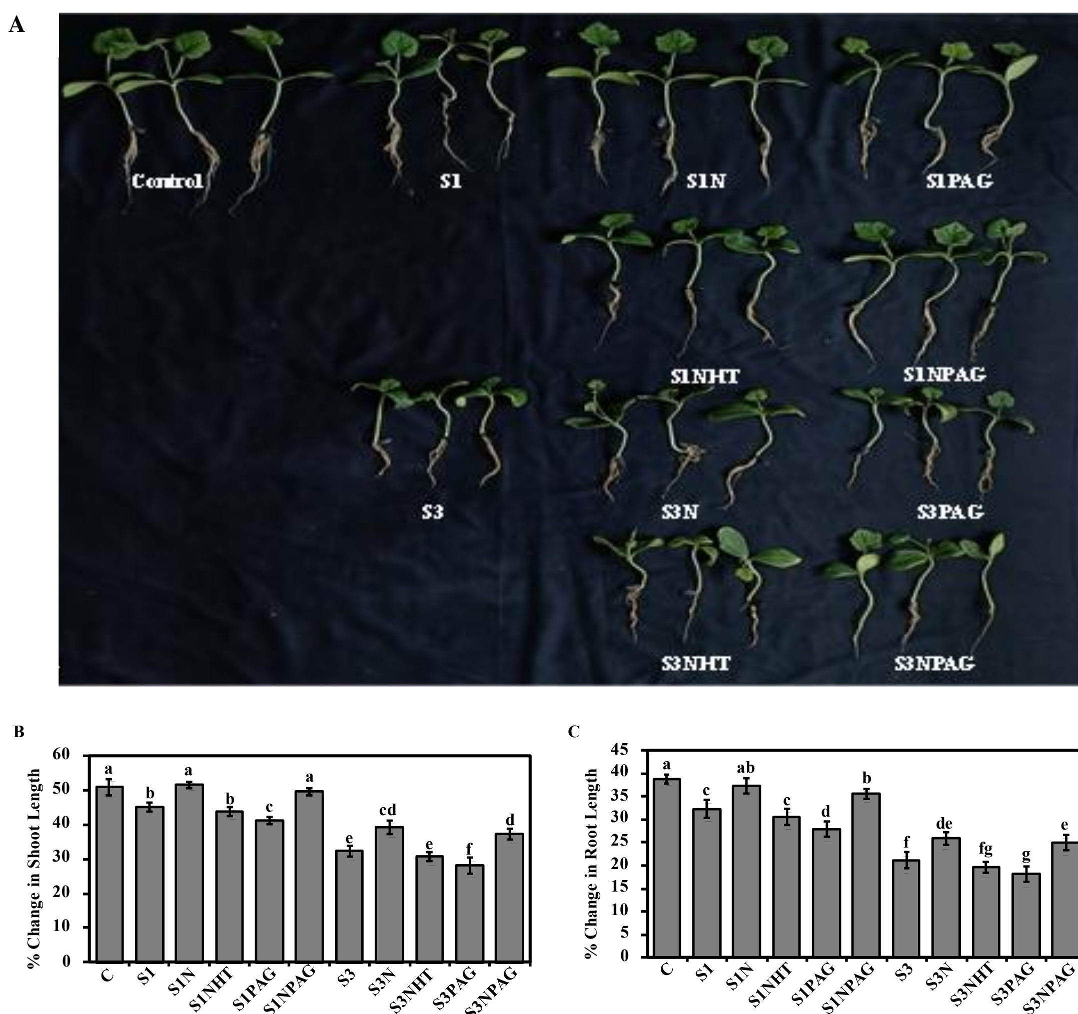

Figure 4.3. (A) The figure depicts the potential of NaHS for maintaining the morphological parameters of salt-stressed sponge gourd. The graphs show the significant rescue of (B) shoot length and (C) root length of salt-stressed sponge gourd on NaHS supplementation.

The hydroponic NaHS treatment was later observed to rescue the salt stress-induced reduction of shoot and root length of sponge gourd (Fig. 4.3B). The shoot length was rescued by 14 and 21% in S1 and S3 stressed seedlings on 50µM NaHS supplementation (Fig. 4.3B). Likewise, NaHS rescued the damage to root length by 16

and 22% in S1N and S3N seedlings, respectively, as compared to their respective salt-stressed seedlings (Fig. 4.3C). Earlier, 200 μM NaHS was reported to improve the shoot-root length of salt-stressed *S. italica* L. (Zhang et al., 2023b). Similarly, treatment with 50 μM NaHS reportedly improved the growth parameters of *T. aestivum* L. during 100 mM NaCl exposure (Deng et al., 2016). NaHS, as an H_2S donor, has been reported to significantly promote root length in a concentration-dependent manner (Zhang et al., 2017). Hence, H_2S supply via NaHS in the present study could have regulated the root meristems, leading to enhanced root elongation, improved nutrient uptake, and subsequently, shoot length in salt-stressed sponge gourd.

Furthermore, to confirm whether NaHS was responsible for rescuing the salt stress-induced morphological changes, S1N and S3N sponge gourds were exposed to scavenger HT. In the presence of HT, the positive effect of NaHS on stressed seedlings was negated (Fig. 4.3A, B). The shoot length of the S1N and S3N treated sponge gourd was reduced by 15 and 22% on S1NHT and S3NHT exposure (Fig. 4.3B). Likewise, the root length was negated on HT exposure and reduced by 18 and 24% in S1NHT and S3NHT, respectively, compared to S1N and S3N sponge seedlings (Fig. 4.3C). HT has been reported to promptly combine with sulfide from exogenously supplied H_2S and generate thiotaurine, thereby reducing the H_2S content (Garcia-Mata and Lamattina, 2010; Mostofa et al., 2015b). Studies have reported the reversal of NaHS induced effects of plant growth parameters under various stresses as in case of NaCl stressed *Malus hupehensis* (Wei et al., 2019), salt stressed *Cucumis sativus* L. (Liu et al., 2022a), lead stressed *Sesamum indicum* (Amooaghaie and Enteshari 2017), *A. thaliana* under manganese stress (Hou et al., 2022), *Medicago sativa* during cadmium stress (Yang et al., 2021), *O. sativa* L. under cadmium stress (Mostofa et al., 2015b), and *Cucurbita pepo* under nickel stress (Valivand and Amooaghaie, 2021a). Thus, in the present study, it is evident that NaHS-mediated H_2S supply was significantly responsible for the salt stress tolerance of sponge gourd.

In addition, to evaluate the function of endogenous H_2S in salt stress alleviation, S1 and S3 seedlings were exposed to an inhibitor of cellular H_2S synthesis, PAG. The PAG, an inhibitor of endogenous H_2S, has been reported to inhibit endogenous H_2S levels (Verma et al., 2023b). PAG supplementation reduced the shoot length of S1 and S3 seedlings by 8 and 13% in S1PAG and S3PAG seedlings, respectively, compared to

their respective S1 and S3 seedlings (Fig. 4.3B). Similar to shoot length, the root length was reduced by 13 and 14% in S1PAG and S3PAG seedlings, respectively (Fig. 4.3C). However, the shoot length was rescued by 20 and 32% in S1NPAG and S3NPAG seedlings than S1PAG and S3PAG seedlings (Fig. 4.3B). Similarly, NaHS supplementation to SPAG seedlings reduced the PAG-induced reduction of root length and enhanced it by 27 and 38% in S1NPAG and S3NPAG seedlings (Fig. 4.3C). PAG reportedly suppresses the enzyme cysteine desulfhydrase responsible for in planta H_2S synthesis, thus halting the production and accumulation of H_2S (Li et al., 2014). Various studies have documented the negative influence of PAG exposure on the morphological and biochemical plant parameters during abiotic stress. The plant growth and stress responses were significantly altered on PAG exposure to NaCl stressed *M. sativa*, *C. sativus* L. (Lai et al., 2014; Luo et al., 2023), Cr (VI) treated *Vigna mungo* L. and *Vigna radiata* L. (Husain et al., 2021), cadmium and arsenate stressed *M. sativa* and *O. sativa* L. seedlings (Mishra and Singh, 2021; Yang et al., 2021), and Ni stressed *C. pepo* (Valivand et al., 2019a).

4.4. NaHS-mediated rescue of photosynthetic pigments, biomass, and carbohydrate accumulation

With the increase in NaCl concentration from S1 to S3, the accumulation of biomass, chlorophyll, carotenoids, and carbohydrate decreased significantly compared to control plants (Fig. 4.4). The chlorophyll a content declined by 13 and 39% in S1 and S3 seedlings, respectively as compared to control (Fig. 4.4A). Likewise, chlorophyll b was reduced by 16 and 42% in S1 and S3 seedlings, respectively (Fig. 4.4B). Similarly, carotenoid content was reduced by 8 and 33% in S1 and S3 sponge seedlings compared to the control (Fig. 4.4C). Salt stress has already been documented to degrade the photosynthetic pigments and reduce the photosynthetic efficiency of plants (Jiang et al., 2019; Yastreb et al., 2020; Rahimi et al., 2021; Muhammad et al., 2021).

Similar changes were observed in the accumulation of fresh weight and carbohydrates in the salt-stressed sponge gourd seedlings (Fig. 4.4D-E). The S1 and S3 seedlings exhibited 17 and 40% decrease in the fresh biomass content as compared to control seedlings (Fig. 4.4D). Likewise, a reduction of 16 and 33% in carbohydrate accumulation as compared to the control was observed on salt stress exposure (Fig.

4.4E). The level of photosynthetic pigments is directly related to the fresh biomass of plants and carbohydrate accumulation (Sharma et al., 2022; Gautam et al., 2024). Hence, the reduced content of chlorophyll and carotenoid in salt-stressed sponge gourd significantly decreased the accumulation of fresh biomass, carbohydrates, and proteins. However, NaHS exposure rescued the chlorophyll a content by 14 and 21% in S1N and S3N seedlings, respectively, compared to S1 and S3 treated samples (Fig. 4.4A). Likewise, chlorophyll b was rescued by 16 and 23% in S1N and S3N seedlings, respectively (Fig. 4.4B). Similarly, carotenoid content was rescued by 9 and 19% by NaHS supplementation to S1 and S3 in S1N and S3N seedlings, respectively (Fig. 4.4C). H_2S-mediated enhancement in the ascorbate peroxidase, superoxide dismutase, and catalase was reported to downregulate oxidative stress to regulate the transcript accumulation of chlorophyll degradation genes, thus promoting the level of photosynthetic pigments and photosynthesis (Li et al., 2014; Kaya and Ashraf, 2019; Gharehbaghli and Sepehri, 2022). Earlier, NaHS exposure has been reported to enhance the accumulation of photosynthetic pigments to induce salt stress tolerance in *Spinacia oleracea* and *O. sativa* L. (Chen et al., 2011; Mostofa et al., 2015a). Consequently, the increased pigment content enhanced the fresh biomass and carbohydrate accumulation of sponge gourd. Therefore, NaHS exposure negated the NaCl-induced decline and rescued the fresh biomass accumulation by 24 and 20% in S1N and S3N seedlings (Fig. 4.4D). NaHS supplementation similarly enhanced carbohydrate content by 29 and 20% in S1N and S3N seedlings compared to S1 and S3 seedlings (Fig. 4.4E). Earlier, NaHS exposure has been reported to enhance the accumulation of photosynthetic pigments, carbohydrates, and proteins to induce salt stress tolerance in *S. oleracea* and *O. sativa* L. (Chen et al., 2011; Mostofa et al., 2015a). Likewise, in the present study, NaHS exposure enhanced the enzyme activities of superoxide dismutase, ascorbate peroxidase, and catalase in salt-stressed sponge gourd, leading to the reduction of oxidative stress markers, hydrogen peroxide, and malondialdehyde. As a result, the degradation of chlorophyll was inhibited, thus rescuing the accumulation of photosynthetic pigments. Enhanced photosynthetic pigments, in turn, improved the fresh biomass and carbohydrate content to induce salt stress tolerance.

Further, the positive effect of NaHS on the pigment content and its associated parameters in salt-stressed sponge gourd seedlings was diminished on scavenger HT

supplementation (Fig. 4.4A-C). The chlorophyll a accumulation declined by 17 and 24% in S1NHT and S3NHT seedlings, respectively, compared to NaHS-treated salt-stressed sponge gourd (Fig. 4.4A). Likewise, chlorophyll b and carotenoids were reduced by 18-27% (Fig. 4.4B) and 12-22% in S1NHT and S3NHT seedlings, respectively (Fig. 4.4C). Similarly, scavenger HT counterbalanced the NaHS-mediated accumulation of fresh weight and carbohydrates in salt-stressed seedlings (Fig. 4.4D-E). The fresh weight and carbohydrate content were reduced by 24-27% and 18-24%, respectively, in S1NHT-S3NHT seedlings compared to S1N and S3N sponge gourd seedlings (Fig. 4.4D-E). The inhibition of photosynthetic pigments and related parameters by HT is ascribed to scavenging of H_2S by HT, which impedes the internal functions of H_2S, as demonstrated by the reduced H_2S levels in the current study. These findings align with previously reported studies in *S. indicum* (Amooaghaie and Enteshari, 2017), *A. thaliana* (Hou et al., 2022), and *O. sativa* L. (Gautam et al., 2022). Similarly, inhibitor PAG reduced the chlorophyll a, chlorophyll b, and carotenoids content by 12-18, 13-20, and 10-15% in S1PAG and S3PAG seedlings, respectively, as compared to S1 and S3 seedlings (Fig. 4.4A-C). The fresh biomass and carbohydrate accumulation were likewise reduced by 14-24% and 6-8% in S1PAG and S3PAG seedlings (Fig. 4.4D-E). In an earlier study, PAG has been reported to reverse the impact of H_2S on pigment, carbohydrate, and fresh biomass in *O. sativa* L. under arsenate stress (Mishra and Singh, 2021).

However, NaHS supplementation minimized the inhibitory effects of PAG and enhanced the accumulation of chlorophyll a, chlorophyll b, and carotenoids by 27-52, 32-56, and 19-42% in S1NPAG and S3NPAG treated sponge gourd seedlings (Fig. 4.4A-C). Likewise, the fresh biomass and carbohydrate content were increased by 32-46% and 26-32% in S1NPAG and S3NPAG seedlings (Fig. 4.4D-E). In salt-stressed sponge gourd seedlings, exogenous H_2S supply has overcome the inhibitory effect of PAG; a similar outcome has been observed in *O. sativa* under arsenate stress (Mishra and Singh, 2021).

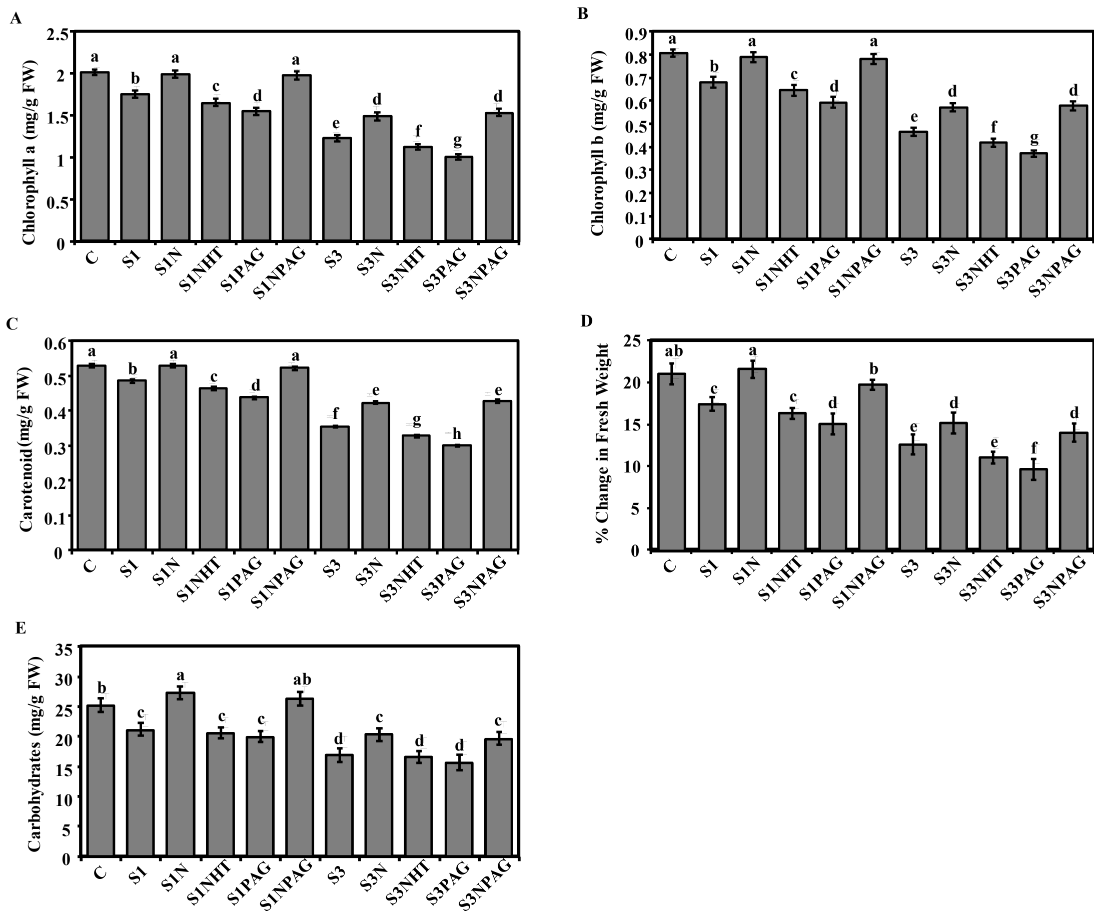

Figure 4.4. Influence of NaCl, NaHS, HT, and PAG on the photosynthetic parameters of sponge gourd. The bar graphs show the considerable enhancement in the levels of (A) chlorophyll a, (B) chlorophyll b, (C) carotenoids, (D) fresh biomass, and (E) carbohydrates in salt-stressed sponge gourd on NaHS exposure.

4.5. NaCl and NaHS mediated altered accumulation of total polyphenols, flavonoids, and antioxidant potential

Total polyphenols and flavonoids are responsible for the antioxidant potential of many vegetable crops. In the present study, salt stress induced a considerable increment of 15 and 31% in the total polyphenolic content of sponge gourd compared to the control (Fig. 4.5A). Similarly, flavonoids were increased by 8 and 22% in S1 and S3 seedlings than the control (Fig. 4.5B). A similar trend was observed in the antioxidant potential of sponge gourd. The antioxidant potential was enhanced by 11 and 29% as compared to the control in S1 and S3 seedlings (Fig. 4.5C). Earlier, salt stress was reported to enhance the phenol, flavonoid, and antioxidant potential of *Carthamus tinctorius* L.

(Golkar and Taghizadeh, 2018). Similarly, 50 and 100 mM NaCl reportedly improved the non-enzymatic antioxidants in *Moringa oleifera* Lam. (Azeem et al., 2023). The upregulation of non-enzymatic antioxidants under stress occurred to enhance the inherent antioxidant potential of the plant to fight against stress (Gill and Tuteja, 2010). Exogenous H_2S exposure is documented to enhance plant antioxidant potential to regulate their stress responses. In the present study, the supplementation of H_2S donor NaHS was observed to enhance the polyphenolic content by 7 and 6% in S1N and S3N, respectively, as compared to S1 and S3 treated seedlings (Fig. 4.5A). Further, flavonoid content was enhanced on NaHS supplementation by 8 and 19% in S1N and S3N sponge gourd as compared to S1 and S3 seedlings (Fig. 4.5B). Likewise, an increment of 9% in the antioxidant potential was evident on NaHS exposure in S1N and S3N seedlings compared to salt-stressed seedlings (Fig. 4.5C). Verma et al. (2023a) have earlier reported the NaHS-mediated increment in the phenol and flavonoid content in salt-stressed *Brassica juncea* L. Likewise, reports have earlier documented the increment in DPPH activity in NaCl-stressed *Helianthus annuus* L. on NaHS exposure (Younis and Mansour, 2023).

The exposure of scavenger HT negated the positive effects of NaHS on the polyphenol, flavonoids, and antioxidant potential of S1N and S3N seedlings (Fig. 4.5A-C). The polyphenolic content was declined by 25 and 19% in S1NHT and S3NHT seedlings than S1N and S3N sponge gourd (Fig. 4.5A). Likewise, the flavonoid accumulation and antioxidant potential of S1NHT and S3NHT seedlings were reduced by 23-29% and 12-13% in comparison to S1N and S2N, respectively (Fig. 4.5B, C). Likewise, the supplementation of S1 and S3 seedlings with inhibitor PAG reduced the polyphenolic content by 20-27%, flavonoids by 23-24%, and antioxidant potential by 7-10% in S1PAG and S3PAG, respectively (Fig. 4.5A-C). Further, supplementation to S1PAG and S3PAG seedlings with NaHS minimized the inhibitory effect of PAG and enhanced the polyphenolic content by 32-43%. Likewise, NaHS exposure increased the flavonoid content and antioxidant potential in S1NPAG and S3NPAG seedlings by 40-52, and 16-21%, respectively, then S1PAG and S3PAG seedlings (Fig. 4.5A-C).

These findings collectively demonstrate the significant role of H_2S in modulating antioxidant responses under salt stress conditions, with NaHS supplementation consistently enhancing protective antioxidant mechanisms in sponge gourd seedlings.

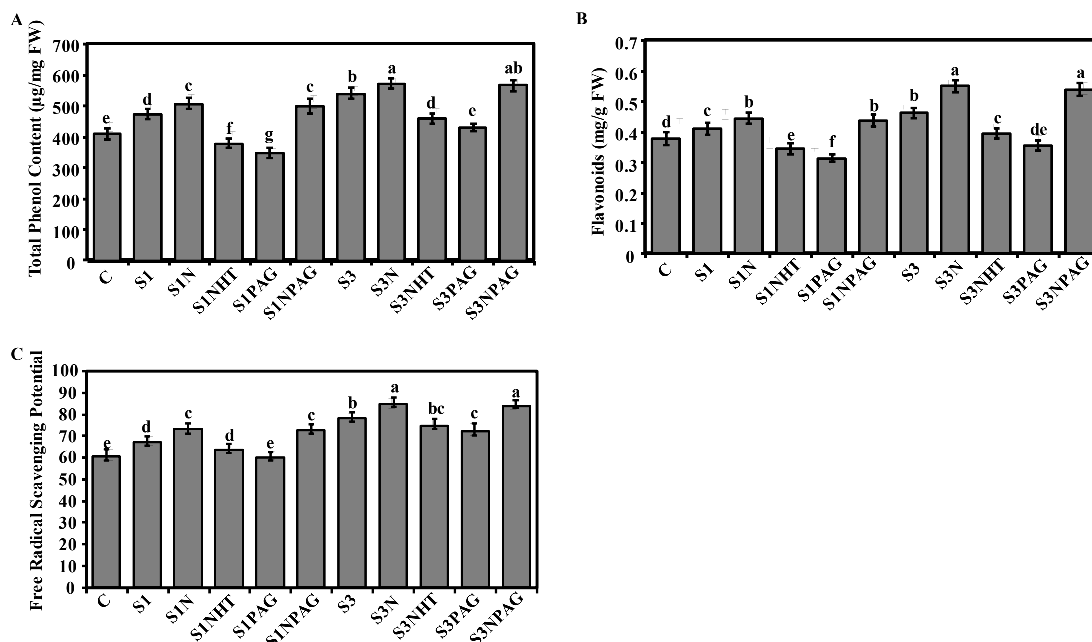

Figure 4.5. The graphs depict the significant alterations in the (A) total phenols and (B) flavonoid accumulation of NaHS-exposed salt-stressed sponge gourd, which lead to (C) an enhanced antioxidant potential on NaHS exposure.

4.6.Altered accumulation of enzymatic antioxidant parameters in sponge gourd

Like the non-enzymatic antioxidant system, the enzymatic antioxidant system was also upregulated in the salt-stressed sponge gourd (Fig. 4.6A-C). The superoxide dismutase activity was considerably increased by 21 and 50% in NaCl-exposed S1 and S3 seedlings compared to the control (Fig. 4.6A). Similarly, the enzyme activity of ascorbate peroxidase was considerably upregulated by 17 and 58% in S1 and S3 seedlings (Fig. 4.6B). Unlike the superoxide dismutase and ascorbate peroxidase, the catalase activity was variously altered with an increase in NaCl concentration. The catalase activity was enhanced by 27% than the control in S1 seedlings, however, the activity was reduced by 12% compared to the control in S3 sponge gourd seedlings (Fig. 4.6C). Similar outcomes have been reported in *V. radiata* L. under salt stress conditions (Ullah et al., 2023). At 50 mM and 100 mM NaCl, superoxide dismutase and ascorbate peroxidase were upregulated; however, CAT activity showed a contrasting response, enhanced at 50 mM but reduced at 100 mM of stress. Likewise, in *Capsicum annuum*, superoxide dismutase and ascorbate peroxidase were enhanced at 100 mM NaCl stress, while catalase was reduced (Kaya et al., 2024). The observed

decline in catalase activity under elevated stress conditions may be attributed to the dissociation of its quaternary structure, specifically the disassembly of its constituent subunits when exposed to higher concentrations of the stressor. This phenomenon aligns with previous findings documented in cadmium-stressed *O. sativa*, where similar enzymatic behavior was reported (Mostofa et al., 2015b).

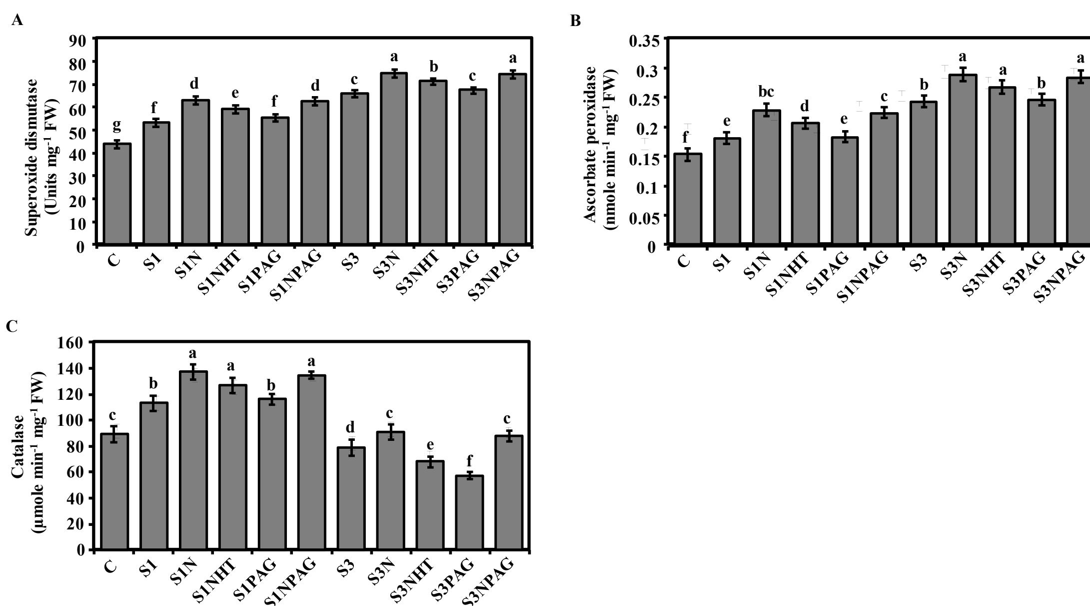

Figure 4.6. Influence of NaCl, NaHS, HT, and PAG on the activities of (A) superoxide dismutase, (B) ascorbate peroxidase, and (C) catalase. NaHS was found to enhance the activities of superoxide dismutase, catalase, and ascorbate peroxidase, thus inducing enhanced salt stress tolerance in sponge gourd.

The exposure of salt-stressed sponge gourd seedlings to NaHS regulated the stress and further enhanced the enzyme activities of superoxide dismutase, ascorbate peroxidase, and catalase. The superoxide dismutase activity was enhanced by 18 and 13% in S1N and S3N seedlings in comparison to S1 and S3 seedlings (Fig. 4.6A). Likewise, the ascorbate peroxidase activity was significantly enhanced by 27 and 19% in S1 and S3 seedlings (Fig. 4.6B). Similarly, exposure of NaHS upregulated catalase activity in both S1N and S3N by 21 and 15% compared to the salt-stressed seedlings (Fig. 4.6C). Similar outcomes have been reported in several other plants, including *C. sativus* L., *T. aestivum* L., and *S. italica* L. (Liu et al., 2022a; Alamer, 2023; Zhang et al., 2023b).

The scavenger HT counteracted the positive impact of NaHS on superoxide dismutase activity and reduced it by 6 and 5% in S1NHT and S3NHT seedlings (Fig. 4.6A). As was observed in superoxide dismutase, the ascorbate peroxidase activity was reduced by 10 and 8% in S1NHT and S3NHT seedlings on scavenger exposure than S1N and S3N seedlings (Fig. 4.6B). Likewise, catalase activity was reduced in S1NHT and S3NHT seedlings by 8 and 25%, respectively, compared to NaHS-exposed salt-stressed sponge gourd (Fig. 4.6C). Similar outcomes have been observed in *M. sativa* under salt and cadmium stress (Lai et al., 2014; Yang et al., 2021*) and T. aestivum* under heat stress (Iqbal et al., 2021).

The introduction of PAG to salt-stressed seedlings elicited differential modulation of antioxidant enzyme activities, revealing a complex enzymatic response. Superoxide dismutase activity exhibited modest upregulation of 4% and 2% in S1PAG and S3PAG seedlings, respectively, when compared to their S1 and S3 salt-stressed counterparts (Fig. 4.6A). Conversely, ascorbate peroxidase activity demonstrated slight decreases of 1-2% in S1PAG and S3PAG seedlings relative to S1 and S3 seedlings (Fig. 4.6B). Catalase activity displayed a differential response pattern to PAG exposure, with a 2% increase observed in S1PAG seedlings compared to S1 seedlings, while exhibiting a significant 27% reduction in S3PAG seedlings relative to S3 seedlings (Fig. 4.6C). Similar outcomes have been observed in *Spartina alterniflora* and *Cyperus malaccensis* under salt stress (Li et al., 2020b) and *M. sativa* under cadmium stress (Yang et al., 2021).

Subsequent exposure to NaHS effectively counteracted PAG-induced alterations, significantly enhancing activities of all antioxidant enzymes. Superoxide dismutase activity increased by 10% and 13% in S1NPAG and S3NPAG seedlings, respectively, compared to S1PAG and S3PAG seedlings (Fig. 4.6A). More pronounced effects were observed in ascorbate peroxidase activity, which demonstrated increases of 16% and 23% in S1NPAG and S3NPAG seedlings relative to S1PAG and S3PAG seedlings (Fig. 4.6B). Notably, catalase activity exhibited the most substantial response to NaHS supplementation, with elevations of 16% in S1NPAG seedlings and a remarkable 53% in S3NPAG seedlings when compared to their respective PAG-treated counterparts (Fig. 4.6C).

These findings demonstrate that NaHS-mediated H_2S exhibits protective effects in modulating enzymatic antioxidant responses against salt-induced oxidative stress in sponge gourd.

4.7. NaHS rescued the hydrogen peroxide and lipid peroxidation content of salt-stressed sponge gourd

The oxidative stress endurance capability was assessed in terms of hydrogen peroxide and malondialdehyde accumulation in sponge gourd seedlings. Salt stress induced significant elevations in oxidative stress markers, with hydrogen peroxide accumulation increased by 77% and 210% in S1 and S3 seedlings, respectively, while malondialdehyde content concurrently rose by 63% and 160% in S1 and S3 salt-stressed sponge gourd seedlings compared to control conditions (Fig. 4.7A-B). Hydrogen peroxide and malondialdehyde content were earlier reported to escalate in *C. annuum*, *Solanum lycopersicum* L., *P. vulgaris* L., and *S. italica* L. on NaCl exposure, which coincides with our findings (Kaya et al., 2024; Ekinci et al., 2023; Yildrim et al., 2023; Zhang et al., 2023b).

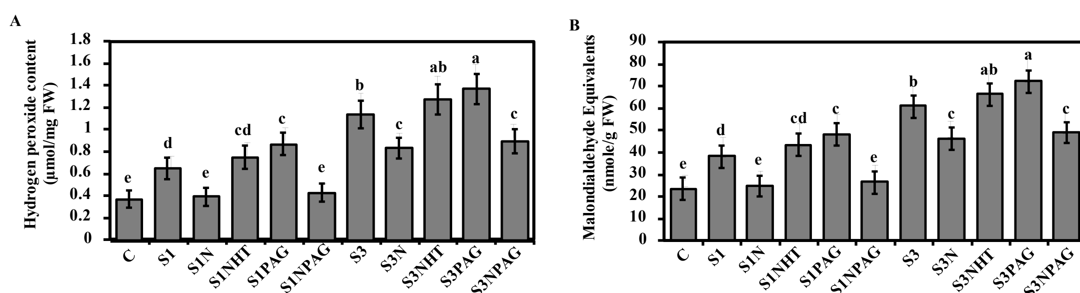

Figure 4.7. The bar graphs depict the influence of NaHS supplementation on the oxidative stress markers (A) hydrogen peroxide, and (B) malondialdehyde, representing lipid peroxidation. NaHS exposure was found to significantly regulate the levels of both hydrogen peroxide and malondialdehyde, thus indicating its potential role in salt stress tolerance of sponge gourd.

Exogenous H_2S exposure is documented to enhance plant antioxidant potential to regulate their stress responses. Likewise, in the current study, the exposure of H_2S donor significantly suppressed the hydrogen peroxide and malondialdehyde accumulation by 39-27% and 35-24%, respectively, in S1N-S3N sponge gourd

seedlings (Fig. 4.7A-B). The decline in hydrogen peroxide content and lipid peroxidation might be due to enhanced enzymatic and non-enzymatic antioxidants by H_2S in sponge gourd under stress. Similarly, earlier studies have documented H_2S-mediated enhancement in the ascorbate peroxidase, superoxide dismutase, and catalase to downregulate oxidative stress (Li et al., 2014; Kaya and Ashraf, 2019; Gharehbaghli and Sepehri, 2022).The oxidative stress mitigatory function of H_2S was further substantiated through experiments involving HT and PAG treatments. The scavenger HT considerably enhanced oxidative stress markers, with hydrogen peroxide content increased by 91% and 53% in S1NHT and S3NHT seedlings, respectively, compared to S1N and S3N seedlings (Fig. 4.7A). Concurrently, malondialdehyde content rose by 76% and 43% in S1NHT and S3NHT seedlings relative to their S1N and S3N counterparts (Fig. 4.7B). Previous research demonstrated that HT intensified oxidative stress in *C. sativus* L. and *A. thaliana* (Qi et al., 2018; Hou et al., 2022; Liu et al., 2022a).

Likewise, PAG exposure enhanced the hydrogen peroxide and malondialdehyde levels. An enhancement of 34 and 21% in hydrogen peroxide accumulation of S1PAG and S3PAG sponge gourd than S1 and S3 salt-stressed seedlings was noticed (Fig. 4.7A). Likewise, PAG exposure enhanced the malondialdehyde levels by 26 and 18% in comparison to S1 and S3 stressed seedlings, in S1PAG and S3PAG seedlings (Fig. 4.7B). PAG induced an increment in hydrogen peroxide and malondialdehyde content in salt-stressed sponge gourd seedlings correlates with earlier studies reported in *O. sativa* L., and *C. malaccensis* (Guo et al., 2017; Li et al., 2020b).

Further, NaHS exposure revoked the effects of PAG on sponge gourd, and hydrogen peroxide content was found to be reduced by 50 and 35% in S1NPAG and S3NPAG seedlings as compared to the S1PAG and S3PAG seedlings (Fig. 4.7A). Likewise, in S1NPAG and S3NPAG seedlings, the malondialdehyde content was reduced by 45 and 32%, respectively, compared to S1PAG and S3PAG seedlings (Fig. 4.7B). These findings suggest that a robust and continuous level of H_2S is required to mitigate the oxidative stress in sponge gourd seedlings.

4.8.Altered protein content in variously treated sponge gourd

NaCl exposure to sponge gourd seedlings considerably reduced the protein content by 14 and 35% compared to control seedlings (Fig. 4.8). A significant influence of NaCl on nitrogen metabolism is noticed in the present study. Similarly, salt stress has been reported to reduce the protein content in *Brassica rapa* L., *Lycopersicon esculentum* Mill., and *C. sativus* L. under salt stress due to enhanced oxidative stress (Kamran et al., 2021; Khan and Alzuaibr, 2022; Liu et al., 2022a). Likewise, protein content is associated with nitrogen metabolism (Farhan et al., 2024). So, it is evident that the inhibition of nitrogen metabolism due to reduced activity of nitrogen metabolizing enzymes in salt-stressed sponge gourd leads to a reduction in protein content.

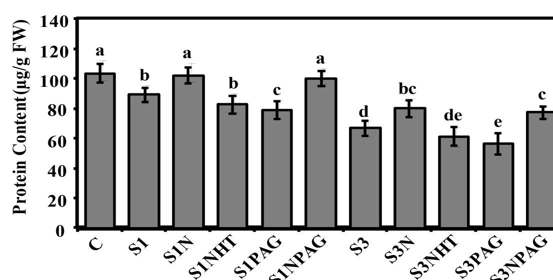

Figure 4.8. The figure shows the effect of NaHS supplementation on the protein accumulation of salt-stressed sponge gourd. The NaCl exposure has reduced the protein content of sponge gourd, but the simultaneous NaHS exposure considerably increased the content. Further, exposure of scavenger HT and inhibitor PAG in addition to NaHS suggested the potential role of H_2S for the observed variation in protein accumulation of sponge gourd.

NaHS supplementation, however, rescued the protein levels and enhanced the accumulation by 14 and 20% in S1N and S3N seedlings, respectively (Fig. 4.8). These findings coincide with earlier reports where H_2S effectively restored the protein content in salt-stressed *B. juncea* L., *Solanum melongena,* and *S. lycopersicum* L. by reducing oxidative stress (Raju and Prasad, 2023; Verma et al., 2023a)

HT scavenger revoked the positive impact of NaHS on protein content and induced a reduction of 19 and 23% in S1NHT and S3NHT seedlings as compared to S1N and S3N seedlings (Fig. 4.8). The inhibitor PAG likewise showed a considerable reduction of 12 and 15% in the protein content of S1PAG and S3PAG seedlings respectively, as compared to their salt stressed seedlings (Fig. 4.8). NaHS exposure however, revoked

the effect of PAG on stressed seedlings and increased the protein content by 27 and 37% in S1NPAG and S3NPAG seedlings than S1PAG and S3PAG seedlings (Fig. 4.8). HT and PAG induced inhibition of H_2S effect on protein content has been reported in *S. melongena* and S. *Lycopersicon* under salt stress (Raju and Prasad, 2021). These findings demonstrate the significant role of H_2S in maintaining protein homeostasis under salt stress conditions in sponge gourd seedlings.

4.9.Effect of varied treatment on nitrogen metabolism of sponge gourd

The process of nitrogen assimilation involves the reduction of nitrate into nitrite via nitrate reductase and then nitrite reductase-mediated reduction of nitrite into ammonium ions. Finally, ammonium ions are assimilated by glutamate synthase in the GS-GOGAT central pathway of nitrogen metabolism and used for protein synthesis (Masclaux-Daubresse et al., 2010; Liu et al., 2022b). Salt stress has been documented to significantly disturb the process of nitrogen metabolism in plants, including nitrification and ammonification, restricting the nitrogen uptake and assimilation (Nazir et al., 2023). Hence, the effect of NaCl, NaHS, HT, and PAG on the nitrogen metabolism and important enzymes involved in nitrogen metabolism was evaluated during the study.

4.9.1. Enhanced inorganic nitrogen content on NaHS supplementation of salt-stressed sponge gourd

NaCl exposure considerably reduced the nitrate and nitrite content in sponge gourd (Fig. 4.9A-B). Nitrate content was reduced by 20 and 45% in S1 and S3 seedlings, respectively, compared to the control (Fig. 4.9A). Likewise, the nitrite content was reduced by 17 and 43% in S1 and S3 sponge gourd, respectively, then the control (Fig. 4.9B). Reports have documented that NaCl-mediated competitive inhibition of nitrate uptake by the transporters, inhibition of nitrate reductase, and other nitrogen metabolizing enzyme activities are responsible for decreased nitrate and nitrite levels during salt stress (Parihar et al., 2021; Raju and Prasad, 2023). So, per the reported studies, the altered activity of nitrate reductase and nitrite reductase, followed by NaCl-mediated decrease in nitrate absorption, is the plausible reason for the reduced nitrate and nitrite levels in salt-stressed sponge gourd.

NaHS supplementation to stressed seedlings, however, enhanced the nitrate and nitrite content by 35-44 and 35-38%, respectively, in S1N and S3N seedlings (Fig. 4.9A-B). Exogenous application of NaHS has previously been documented to enhance nitrate and nitrite uptake across various plant species (Rizwan et al., 2019; Raju and Prasad, 2023).

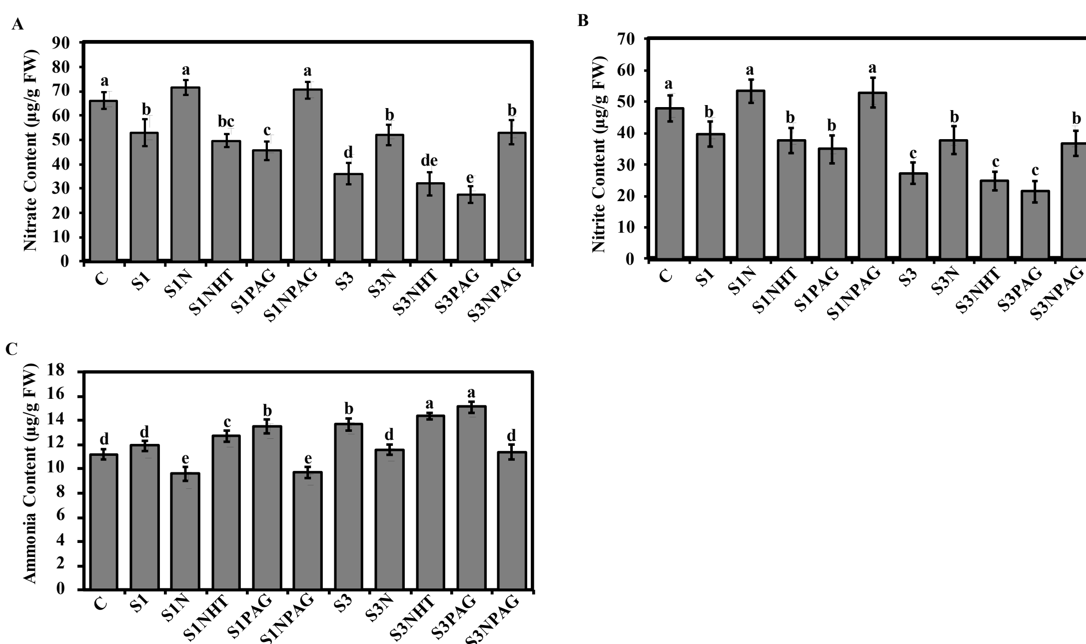

Figure 4.9. Effect of NaCl, NaHS, HT, and PAG on the metabolites of nitrogen metabolism. The bar graphs show the considerable enhancement in the accumulation of (A) nitrate and (B) nitrite in salt-stressed sponge gourd on NaHS exposure. However, NaHS exposure was found to reduce the (C) ammonia levels of salt-stressed sponge gourd, indicating stress tolerance.

The HT exposure to NaHS exposed seedlings inhibited the nitrate accumulation and reduced it by 30 and 38% in S1NHT and S3NHT seedlings (Fig. 4.9A). Likewise, the nitrite accumulation was reduced by 29 and 34% in S1NHT and S3NHT seedlings than S1N and S3N seedlings, respectively (Fig. 4.9B). A similar outcome has been observed in *C. annuum* under drought stress (Kaya and Shabala, 2023). Further, PAG exposure of S1-S3 treated sponge gourd reduced the nitrate and nitrite content by 14-24% and 12-21%, respectively, in S1PAG-S3PAG sponge gourd seedlings (Fig. 4.9A-B). However, NaHS supplementation of PAG exposed seedlings rescued the reduction of nitrate accumulation and enhanced the content by 55 and 93% in S1NPAG and S3NPAG sponge gourd seedlings (Fig. 4.9A). Similarly, NaHS exposure was found to

rescue the PAG-induced nitrite reduction by 51 and 70% in S1NPAG and S3NPAG sponge gourd seedlings (Fig. 4.9B).

On the contrary, ammonia accumulation showed a varied trend. The ammonia content was considerably enhanced by 6 and 22% in salt-stressed S1 and S3 seedlings than the control (Fig. 4.9C). Increased proteolysis and reduced activity of glutamine synthetase and glutamate synthase have been documented as responsible for enhanced ammonia accumulation in salt-stressed *P. Vulgaris*, *S. melongena*, and *S. lycopersicum* L, and a similar response pattern was reported in the present study (Ozfidan-Konakci et al., 2020; Raju and Prasad, 2023). Furthermore, increased ammonium levels have been associated with reduced plant morphology. So, it is evident that the inhibition of ammonium assimilation due to reduced glutamine synthetase and glutamate synthase activity in salt-stressed sponge gourd enhanced the stress and significantly reduced their morphological parameters in the present study.

On NaHS exposure, the ammonia accumulation was counteracted by 19 and 15% in S1N and S3N seedlings, then salt-stressed seedlings (Fig. 4.9C). Previous research documented that 40 µM NaHS treatment decreased ammonia content in *S. melongena* and *S. lycopersicum* L. under 20 mM NaCl stress conditions through enhanced GS-GOGAT pathway performance (Raju and Prasad, 2023).

The scavenger HT, negated the NaHS-induced reduction of ammonia, and the content was then enhanced by 32 and 24% in S1NHT and S3NHT seedlings, respectively (Fig. 4.9C). The exposure of inhibitor PAG to NaCl stressed seedlings further enhanced the ammonia content by 13 and 10% in S1PAG and S3PAG (Fig. 4.9C). Furthermore, NaHS exposed to S1PAG and S3PAG reduced ammonia accumulation by 28 and 25% in S1NPAG and S3NPAG seedlings, respectively (Fig. 4.9C). A similar outcome has been observed in *C. annuum* under drought stress (Kaya and Shabala, 2023) and *S. melongena* and *S. Lycopersicon*. Under salt stress (Raju and Prasad, 2023). This finding demonstrates the crucial role of H_2S in regulating inorganic nitrogen content in sponge gourd.

4.9.2. NaHS altered enzymes involved in nitrogen metabolism: nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, and glutamate dehydrogenase.

The activity of nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, and glutamate dehydrogenase was evaluated in the sponge gourd exposed to NaCl, NaHS, HT, and PAG. The nitrate and nitrite reductase activities were inhibited in sponge gourd seedlings on NaCl exposure. The nitrate reductase activity was reduced by 14 and 38%, and nitrite reductase activity by 9 and 31% in S1 and S3 seedlings, respectively, than control seedlings (Fig. 4.10A-B). Under 100 mM NaCl stress, nitrate reductase activity has been reported to reduce in *T. aestivum* L. and *V. radiata* L. (Alamer, 2023; Kumari et al., 2023; Ullah et al., 2023). Similarly, under 20 mM of NaCl stress, nitrite reductase activity was reported to be reduced in *S. melongena* and *S. Lycopersicon* L. (Raju and Prasad, 2023). The decline in nitrate and nitrite reductase activity is attributed to decreased nitrate uptake and enhanced hydrogen peroxide production (Rizwan et al., 2019).

The S1 and S3 seedlings, when treated with NaHS, showed 17 and 18% upregulation in the nitrate reductase activity and 13 and 14% in the nitrite reductase activity, respectively, in S1N and S3N seedlings (Fig. 4.10A-B). Exogenous NaHS application has been documented to enhance both nitrate reductase and nitrite reductase activity across diverse plant species (Rizwan et al., 2019; Raju and Prasad, 2023). This beneficial effect on nitrate reductase activity under salt stress conditions has been specifically observed in *Betula platyphylla*, *Cyclocarya paliurus*, and *P. vulgaris* L. (Ma et al., 2019; Chen et al., 2021b; Dawood et al., 2022a).

However, HT treatment of the NaHS exposed salt stress seedlings reverted the enhancement in the nitrate reductase and nitrite reductase activity and 17 and 18% reduction in the nitrate reductase and 13 and 16% reduction in the activity of nitrite reductase were observed in S1NHT and S3NHT seedlings (Fig. 4.10A-B). A similar outcome has been observed in *L. esculentum* Mill. under salt stress and *C. annuum* under drought stress (Khan and AlZuaibr, 2022; Kaya and Shabala, 2023). PAG exposure inhibited the nitrate reductase and nitrite reductase activity by 8-9% and 6-9%, respectively, in S1PAG and S3PAG seedlings relative to S1 and S3 salt-stressed

seedlings (Fig. 4.10A-B). A similar outcome has been reported in *S. melongena* and *S. Lycopersicon* under salt stress (Raju and Prasad, 2023).

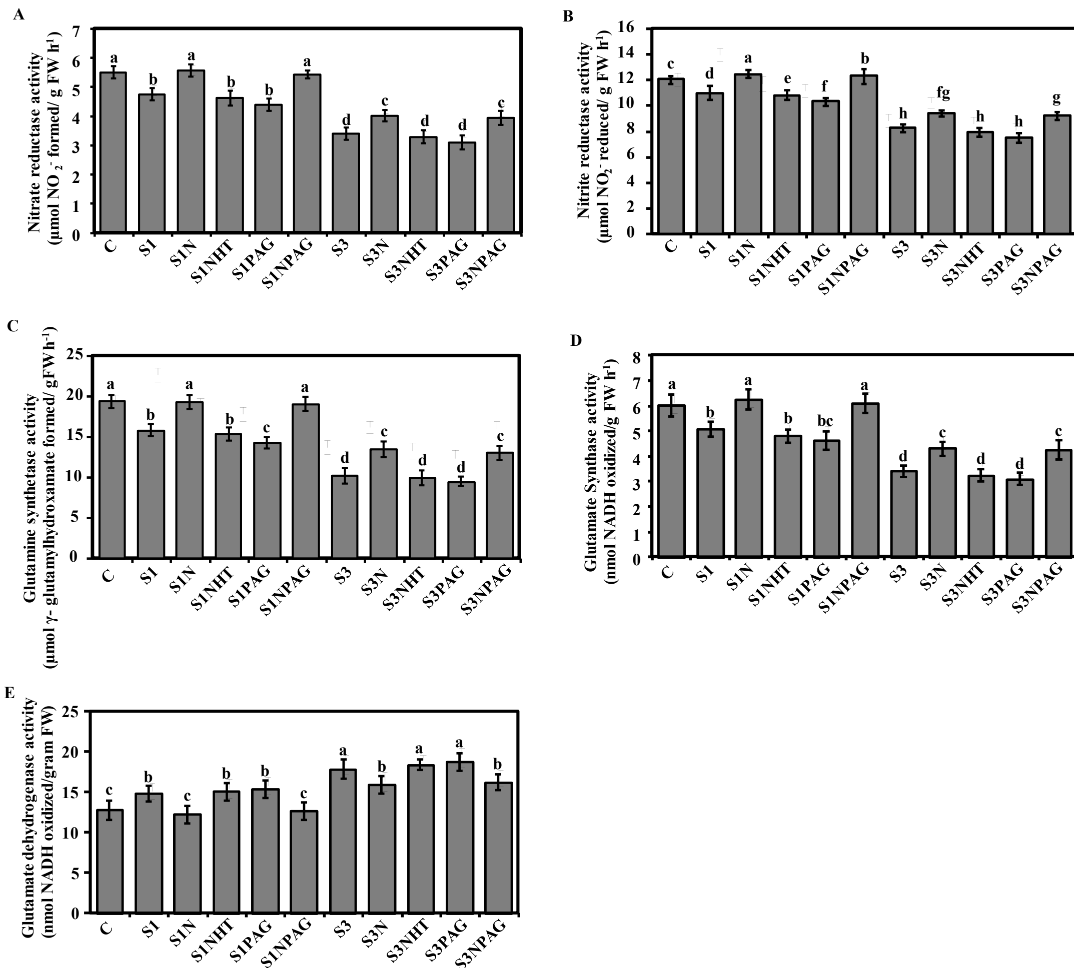

Figure 4.10. Effect of NaCl, NaHS, HT, and PAG on the enzymes of nitrogen metabolism. The graphs represent NaHS-mediated enhanced activity of (A) nitrate reductase, (B)nitrite reductase, (C) glutamine synthetase, and (D) glutamate synthase in salt-stressed sponge gourd, indicating NaHS-mediated rescue of nitrogen metabolism under stressed conditions. On the contrary, the activity of (E) glutamate dehydrogenase was considerably reduced on NaHS exposure, indicating its potential role in stress regulation of sponge gourd.

In addition, NaHS exposure was observed to rescue the nitrate and nitrite reductase activity. The activities of nitrate and nitrite reductase were enhanced by 24-28% and 19-22%, respectively, in S1NPAG-S3NPAG sponge gourd than S1PAG and S3PAG seedlings (Fig. 4.10A-B). HT and PAG inhibit the production of endogenous H_2S in salt-stressed sponge gourd, thereby reducing the nitrate and nitrite reductase activity.

However, exogenous H_2S exposure to sponge gourd seedlings overcame the inhibitory effect of PAG and improved the activities of nitrate and nitrite reductase, respectively. Similar to nitrate and nitrite reductase, NaCl exposure inhibited the glutamine synthetase and glutamate synthase activity in sponge gourd. The glutamine synthetase and glutamate synthase activity were reduced by 19-47 and 16-43% in sponge gourd seedlings exposed to S1 and S3 NaCl concentrations compared to the control (Fig. 4.10C-D). These findings align with reported inhibition of glutamine synthetase and glutamate synthase activity in *V. radiata* L. and *L. esculentum* Mill. under NaCl stress (Ullah et al., 2023; Khan and AlZuaibr, 2022). The decreased activity of glutamine synthetase and glutamate synthase can be attributed to their oxygen-labile nature and the elevated production of ROS under NaCl stress, as previously demonstrated by Raju and Prasad (2023).

NaHS treatment increased the glutamine synthetase activity by 22 and 31% in S1N and S3N seedlings compared to S1 and S3 seedlings. Likewise, glutamate synthase activity was improved by 23 and 26% in S1N and S3N seedlings, respectively, compared to S1 and S3 sponge gourd seedlings (Fig. 4.10C-D). H_2S exposure has earlier been documented to promote the activity of glutamine synthetase and glutamate synthase in *S. melongena* and *S. Lycopersicon* L. (Raju and Prasad, 2023).

The scavenger HT decreased the glutamine synthetase activity by 20- 26%, and the glutamate synthase activity by 23- 25% in the S1NHT-S3NHT seedlings, respectively, compared to the S1N and S3N treated sponge gourd (Fig. 4.10C-D). A similar outcome has been observed in *L. esculentum* Mill. under salt stress and *C. annuum* under drought stress (Khan and AlZuaibr, 2022; Kaya and Shabala, 2023).

Additionally, adding PAG to the S1-S3 salt-stressed seedlings lowered the glutamine synthetase activity and the glutamate synthase activity by 9-7% and 9%, respectively (Fig. 4.10C-D). However, NaHS helped to reverse the negative impact of PAG and increased the glutamine synthetase and glutamate synthase activity in the S1NPAG and S3NPAG seedlings, respectively (Fig. 4.10C-D). These findings demonstrate the protective effect of H_2S in regulating nitrogen assimilating enzymes.

Glutamate dehydrogenase activity, however, exhibited a trend contrary to other nitrogen metabolizing enzymes, demonstrating an inverse response pattern compared to the previously described nitrogen assimilation pathway enzymes. The glutamate

dehydrogenase activity was upregulated by 15 and 39% in S1 and S3 seedlings than the control (Fig. 4.10E). Literature documents that higher ammonium levels act as an indicator of stress in plants, which leads to increased glutamate production by the enhanced activity of glutamate dehydrogenase under stress (Skopelitis et al., 2006; Masclaux-Daubresse et al., 2010).

NaHS reduced the glutamate dehydrogenase activity by 17 and 11% in S1N and S3N sponge gourd in comparison to salt stressed seedlings (Fig. 4.10E). This could be due to the restoration of the GS-GOGAT pathway by NaHS supplementation to stressed seedlings (Raju and Prasad, 2023).

HT, however, revoked the impact of NaHS on glutamate dehydrogenase activity and enhanced it by 23 and 15% in S1NHT and S3NHT seedlings (Fig. 4.10E). In addition, PAG exposure also increased the glutamate dehydrogenase activity in S1PAG and S3PAG by 4 and 5%, than S1 and S3 salt-stressed seedlings (Fig. 4.10E). Further exposure of NaHS to S1PAG and S3PAG seedlings reduced the glutamate dehydrogenase activity by 18 and 13% in S1NPAG and S3NPAG seedlings, respectively (Fig. 4.10E).

H_2S exposure has been documented to promote the activity of nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase, finally leading to enhanced content of inorganic nitrogen and proteins (Raju and Prasad, 2023). The present investigation demonstrates that H_2S supplementation through NaHS application enhanced nitrate uptake and nitrogen assimilating enzyme activities in salt-stressed sponge gourd seedlings, effectively regulating ammonium levels and consequently promoting plant growth under adverse salinity conditions.

The observed morphological and biochemical evaluations thus indicate that NaHS was potentially able to regulate the salt stress responses of sponge gourd. NaHS has been documented to regulate the endogenous H_2S levels of plants as it is a H_2S donor (Iqbal et al., 2021; Tang et al., 2020; Husain et al., 2022), hence, the H_2S levels of variously treated sponge gourd were also evaluated in the present study.

4.10. H_2S content augmented on NaHS exposure

H_2S is known to regulate the plant responses to induce salt stress tolerance. Hence, the H_2S accumulation of sponge gourd seedlings exposed to NaCl, NaHS, HT, and PAG was evaluated in the present study. NaCl treatment was found to considerably increase the H_2S content by 13 and 22% in S1 and S3 treated sponge gourd seedlings compared to the control (Fig. 4.11). H_2S accumulation serves as a critical signaling molecule in plant stress response mechanisms, specifically inducing salt stress tolerance as documented in recent investigations (Yang et al., 2023; Zhao et al., 2023). Similarly, in the present investigation, the increment of H_2S content under NaCl stress in sponge gourd proves its role as a stress signaling molecule.

NaHS functions as an established H_2S donor capable of modulating plant responses to abiotic stressors (Li et al., 2014; Min et al., 2016). In the present study, exogenous NaHS application induced significant increases of 12 and 11% in endogenous H_2S accumulation in S1N and S3N seedlings, respectively, compared to their S1 and S3 counterparts (Fig. 4.11). Plants exposed to various abiotic stresses like heat, drought, and salt have been reported to alleviate the stress on exogenous NaHS exposure due to enhanced H_2S content (Min et al., 2016; Ding et al., 2019; Du et al., 2022; Yang et al., 2023; Zhang et al., 2023b). Exogenous NaHS was documented to improve the endogenous H_2S levels and enhance the photosynthesis, antioxidant parameters of the treated plants to regulate the amount of hydrogen peroxide and malondialdehyde, thus leading to regulation of abiotic stress-induced oxidative stress (Min et al., 2016; Ding et al., 2019; Du et al., 2022; Yang et al., 2023; Zhang et al., 2023b). Therefore, as observed in the present study, the increased H_2S level of NaHS-exposed sponge gourd was responsible for the improved morphological, biochemical, and antioxidant parameters to alleviate the salt stress responses and tolerate salt stress.

The HT, scavenger of exogenous H_2S, and PAG, an inhibitor of endogenous H_2S, have been reported to inhibit endogenous H_2S levels (Verma et al., 2023b). Similarly, in the present study, in the presence of HT and PAG, the H_2S content was significantly reduced. The reduction of 32 and 45% in H_2S accumulation of S1NHT and S3NHT compared to S1N and S3N seedlings was evident (Fig. 4.11). HT has been reported to promptly combine with sulfide from exogenously supplied H_2S and generate thiotaurine (Mostofa et al., 2015b; Garcia-Mata and Lamattina, 2010). Studies have

reported the reversal of NaHS induced effects on plant growth parameters under various stresses as in case of NaCl stressed *M. hupehensis* (Wei et al., 2019), salt stressed *C. sativus* L. (Liu et al., 2022a), lead stressed *S. indicum* (Amooaghaie and Enteshari 2017), *A. thaliana* under manganese stress (Hou et al., 2022), *M. sativa* during cadmium stress (Yang et al., 2021), *O. sativa* L., under cadmium stress (Mostofa et al., 2015b), and *C. pepo* under nickel stress (Valivand and Amooaghaie, 2021a). Thus, in the present study, it is evident that NaHS-mediated H_2S supply was significantly responsible for the salt stress tolerance of sponge gourd.

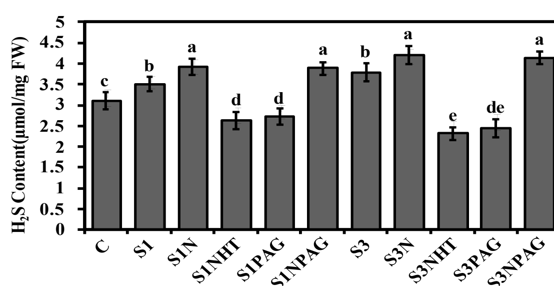

Figure 4.11. NaHS is considered a probable H_2S donor. The bar graph shows the enhanced accumulation of endogenous H_2S in salt-stressed sponge gourd on NaHS exposure. So, hydroponic exogenous exposure of NaHS was a successful mode of ameliorating H_2S levels in sponge gourd to induce salt stress tolerance.

Likewise, H_2S content was reduced by 22 and 36% in S1PAG and S3PAG seedlings in comparison to salt-stressed sponge gourd (Fig. 4.11). Similarly, PAG reportedly suppresses the enzyme cysteine desulfhydrase responsible for in planta H_2S synthesis, thus halting the production and accumulation of H_2S (Li et al., 2014). Various studies have documented the negative influence of PAG exposure on the morphological and biochemical plant parameters during abiotic stress. The plant growth and stress responses were significantly altered on PAG exposure to NaCl stressed *M. sativa*, *C. sativus* L. (Lai et al., 2014; Luo et al., 2023), Cr (VI) treated *V. mungo* L. and *V. radiata* L. (Husain et al., 2021), cadmium and arsenate stressed *M. sativa* and *O. sativa* L. seedlings (Mishra and Singh, 2021; Yang et al., 2021), and Ni stressed *C. pepo* (Valivand et al., 2019a).

NaHS supplementation effectively counteracted the PAG-induced suppression of endogenous H_2S production, and hence significantly elevated H_2S content by 43% and

70% in S1NPAG and S3NPAG sponge gourd seedlings, respectively, compared to their S1PAG and S3PAG counterparts (Fig. 4.11). The improved H_2S in sponge gourd seedlings by NaHS exposure ultimately results in enhanced morphological, biochemical, and antioxidant parameters of these seedlings. These findings indicate that the H_2S exogenous supply is necessary for inducing salt stress tolerance in sponge gourd in the present study. Supplementation of NaHS considerably enhanced H_2S availability, thereby supporting the sponge gourd growth under salt stress conditions.

NaHS-mediated H_2S supply to plants is a good strategy for promoting abiotic stress tolerance in plants. NaCl exposure considerably reduced the root length probably due to inhibition of meristematic zone activity, which reduced the nutrient supply to above-ground plant parts, leading to shorter plants than non-stressed plants. Salt stress enhanced the oxidative stress in sponge gourd, inducing the degradation of photosynthetic pigments, followed by the degradation of carbohydrates and proteins. Further, NaCl reduced the nitrate uptake and absorption due to competitive inhibition, and enhanced ammonium levels, thus augmenting stress and reducing plant growth and development. NaHS-mediated H_2S supply reversed the inhibitory effects of NaCl and promoted the growth and stress tolerance of sponge gourd. NaHS enhanced the activity of superoxide dismutase, catalase, and ascorbate peroxidase, leading to the scavenging of oxidative stress, thus inhibiting the degradation of chlorophyll. Rescue of the photosynthetic pigment content induced the accumulation of carbohydrates and proteins in salt-stressed sponge gourd. Further, NaHS promoted the transport-mediated uptake of nitrates, and enhanced the nitrogen metabolizing enzymes to decrease ammonium levels, thus positively influencing the nitrogen metabolism. This influence overall promoted plant growth, thus mitigating salt stress in sponge gourd.

The effect of salt stress on okra was also devastating. Salt stress severely impairs its development and yield. However, no studies have examined NaHS effects on salt-stressed okra seedlings to elucidate the role of H_2S for stress tolerance in okra. Therefore, in this study underlying mechanism of H_2S in okra's salt stress tolerance by analyzing morphological and biochemical parameters in hydroponically-grown salt-stressed okra seedlings was evaluated with particular focus on nitrogen metabolism impacts.

4.11. Determination of salt concentrations to be used for the okra study

In the pioneer experiment, seedlings were transferred to a hydroponic system and exposed to three different concentrations of NaCl, 25 mM (S1), 50 mM (S2), and 75 mM (S3) for seven days. The treated seedlings underwent comprehensive morphological and biochemical analyses to establish the optimal NaCl concentration for subsequent experiments (Table 4.4). With the increase in the NaCl concentration from S1 to S3, the shoot length of seedlings was reduced by 15, 28, and 49%, in contrast to the control (Table 4.4; Fig. 4.12). Likewise, the root depicted a significant drop in its length on NaCl exposure by 24, 43, and 61% in S1, S2, and S3 seedlings, respectively, in contrast to the control (Table 4.4; Fig. 4.12).

Figure 4.12. The figure illustrates the impact of NaCl on the morphological attributes of okra seedlings.

Fresh weight buildup of okra was also reduced when the salt content was changed from 25 to 75 mM, as shown in Table 4.4. Thus, the results indicated that an increase in NaCl concentration considerably reduced the morphological parameters of okra. Additionally, when the concentration of NaCl increased, the assessed biochemical parameters, including chlorophyll a, chlorophyll b, carotenoid, carbohydrates, and protein content, were noticeably decreased in salt-stressed okra compared to the control. NaCl stress was observed to increase the total phenolics accumulation by 22, 38 and 57% in S1, S2, and S3 okra seedlings compared to the control seedlings (Table 4.4). Similarly, the flavonoid levels were also enhanced by 21, 38, and 52% than the control in salt-stressed seedlings (Table 4.4). Likewise, the activity of antioxidant enzymes

SOD, CAT and APX also showed increment with increase in NaCl concentration. The SOD activity of S1, S2 and S3 okra seedlings was enhanced by 30, 46, and 57%, respectively, in comparison to the control seedlings (Table 4.4). A similar trend was observed in the APX and CAT activity of salt-stressed okra seedlings. An increment of 32-73 and 37-84% was observed in the APX and CAT activity in NaCl-treated okra seedlings compared to the control (Table 4.4). Studies have earlier reported that the enhanced accumulation of phenol, flavonoids, and enzymatic activities under stress led to the enhanced antioxidant potential under NaCl stress (Huang et al., 2024). Likewise, the antioxidant potential of salt-stressed okra seedlings was enhanced by 9-31% with the increase in NaCl concentration than the control (Table 4.4).

Okra showed hyperaccumulation of hydrogen peroxide and malondialdehyde levels, suggesting that oxidative stress augmented as the salt content increased from S1 to S3 (Table 4.4). Malondialdehyde content was notably enhanced by 75, 131, and 177% in S1, S2, and S3 okra seedlings relative to the control (Table 4.4). Similar to malondialdehyde content, an increment of 91, 165, and 235% was observed in the hydrogen peroxide level for S1, S2, and S3 okra seedlings, respectively, compared to the control (Table 4.4). Escalation of NaCl concentration coincides with the abrupt rise in ROS. A dramatic rise in hydrogen peroxide and malondialdehyde content under NaCl stress suggested oxidative stress, indicating compromised membrane integrity, and the occurrence of lipid peroxidation in salt-stressed okra seedlings. Therefore, in the present investigation, the lowest and maximum doses of NaCl, 25 mM (S1) and 75 mM (S3), were employed for further experiments.

Table 4.4. Effects of different NaCl concentrations on okra. Elevated NaCl concentrations drastically diminished the morphological and biochemical parameters under investigation.

Parameters	C	S1	S2	S3
Fresh weight	58.48±2.20[a]	47.27±1.55[b]	31.91±2.63[c]	22.83±2.18[d]
Shoot Length	38.94±1.13[a]	33.03±1.53[b]	28.10±2.53[c]	19.88±1.18[d]
Root Length	62.35±2.83[a]	47.05±2.62[b]	35.60±2.22[c]	24.26±2.42[d]
Chlorophyll a	1.77±0.03[a]	1.58±0.04[b]	1.37±0.03[c]	1.16±0.04[d]
Chlorophyll b	0.69±0.012[a]	0.59±0.02[b]	0.49±0.01[c]	0.43±0.01[d]
Carotenoids	0.47±0.0055[a]	0.43±0.0053[b]	0.38±0.0046[c]	0.33±0.0055[d]
Carbohydrates	19.44±0.69[a]	14.59±0.71[b]	12.96±0.54[c]	10.61±0.64[d]
Protein	129.59±3.13[a]	99.12±3.25[b]	84.89±3.17[c]	64.83±2.67[d]
Hydrogen peroxide	0.33±0.03[d]	0.63±0.040[c]	0.88±0.037[b]	1.11±0.04[a]
Malondialdehyde equivalents	11.91±1.81[d]	20.90±1.02[c]	27.57±1.41[b]	33.03±2.24[a]
Total phenolic content	225.5±7.97[d]	274.72±5.1[c]	310.81±10.33[b]	355.5±7.5[a]
Flavonoids	0.49±0.021[d]	0.59±0.019[c]	0.67±0.018[b]	0.74±0.017[a]
Free radical scavenging potential	68.95±2.43[d]	75.03±1.53[c]	83.30±1.73[b]	90.55±2.19[a]
Superoxide dismutase	54.81±1.14[d]	71.57±1.44[c]	79.88±1.78[b]	86.03±1.86[a]
Ascorbate peroxidase	0.28±0.012[d]	0.37±0.018[c]	0.43±0.016[b]	0.49±0.018[a]
Catalase	108±5.29[d]	148±6[c]	169.3±7.02[b]	199.3±4.16[a]

★a, b, c, d indicates significant differences

4.12. Determination of NaHS concentrations for the okra study

Similarly, optimal NaHS concentration was established through okra seedling exposure to five distinct NaHS concentrations: 50 µM (N1), 100 µM (N2), 150 µM (N3), 200 µM (N4), and 250 µM (N5) in a hydroponic system. The treated seedlings underwent morphological and biochemical evaluation to ascertain the optimal NaHS concentration (Table 4.5). Okra seedlings treated with N1, N2, N3, N4, and N5 showed 43, 49, 42,

38, and 33% increase in shoot elongation, respectively, however, the elongation was only 38% in untreated control seedlings (Table 4.5; Fig. 4.13). Similarly, when Okra was exposed to N1, N2, N3, N4, and N5 NaHS concentrations, the root length was increased by 74, 82, 71, 64, and 58% compared to a 61% increase in the control group (Table 4.5; Fig. 4.13). A similar trend in fresh weight augmentation of N1-N5 treated okra seedlings was observed (Table 4.5). Thus, the optimal NaHS concentration was determined to be N2 (100 µM) based on the evaluation of morphological features.

Figure 4.13. The figure illustrates the potential of NaHS for maintaining the morphological parameters of the okra.

Similarly, the biochemical parameters were assessed both with and without NaHS exposure. Table 4.5 shows that in N1-N5 okra seedlings, the carbohydrates, proteins, and enzymatic and non-enzymatic antioxidant characteristics showed enhanced accumulation than the control. However, among all treated seedlings, N2 okra seedlings depicted the highest % of carbohydrates, proteins, and enzymatic and non-enzymatic antioxidants compared to the control (Table 4.5). Similarly, levels of hydrogen peroxide and malondialdehyde were maximally decreased by N2 exposure compared to control and other NaHS concentrations (Table 4.5). Therefore, for further investigation, a concentration of 100 µM (N2) was utilized, as revealed by the morphological and biochemical analysis of okra treated with NaHS.

Table 4.5. Effect of NaHS exposure on okra. N2 (100 µM) improved the measured morphological and biochemical plant characteristics.

Parameters	C	N1	N2	N3	N4	N5
Fresh weight	56.56±1.96^{d}	67.37±1.31^{b}	74.85±1.66^{a}	64.53±1.03^{c}	58.59±1.93^{d}	50.62±0.99^{e}
Shoot Length	38.06±0.78^{d}	43.67±1.32^{b}	49.37±1.59^{a}	41.72±1^{c}	38.38±0.99^{d}	32.85±0.52^{e}
Root Length	61.20±1.25^{d}	74.17±2.66^{b}	82.33±1.08^{a}	71.24±1.30^{c}	64.20±1.48^{d}	57.64±1.16^{e}
Chlorophyll a	1.79±0.042^{d}	2.06±0.037^{b}	2.17±0.006^{a}	1.93±0.047^{c}	1.75±0.031^{d}	1.61±0.0493^{e}
Chlorophyll b	0.73±0.017^{d}	0.87±0.022^{b}	0.92±0.019^{a}	0.82±0.017^{c}	0.75±0.014^{d}	0.69±0.021^{e}
Carotenoids	0.46±0.009^{d}	0.54±0.011^{b}	0.57±0.010^{a}	0.51±0.012^{c}	0.47±0.014^{d}	0.43±0.014^{e}
Carbohydrate	18.52±0.54^{d}	22.30±0.75^{b}	24.46±0.68^{a}	20.69±0.61^{c}	19.40±0.53^{d}	16.82±0.63^{e}
Protein	133.28±3.94^{e}	164.29±4.90^{b}	176.08±4.11^{a}	153.76±4.46^{c}	141.08±5.80^{d}	122.69±3.14^{f}
Hydrogen peroxide	0.33±0.016^{b}	0.24±0.020^{c}	0.18±0.020^{d}	0.28±0.016^{c}	0.35±0.024^{b}	0.40±0.016^{a}
Malondialdehyde Equivalents	11.18±0.84^{b}	8.60±0.41^{d}	6.88±0.39^{e}	9.80±0.68^{c}	11.09±0.68^{b}	12.47±0.86^{a}
Total phenolic content	228±3.54^{e}	268.62±5.41^{b}	289.87±4.16^{a}	250.03±5.21^{d}	224.87±6.03^{e}	260.03±5.01^{c}
Flavonoids	0.48±0.012^{d}	0.57±0.011^{b}	0.63±0.013^{a}	0.53±0.012^{c}	0.49±0.009^{d}	0.56±0.010^{b}
Free radical scavenging potential	67.59±1.15^{d}	77.31±2.07^{b}	82.16±1.72^{a}	72.54±1.28^{c}	67.03±1.29^{d}	75.46±1.05^{b}

Superoxide dismutase	56.40±2.05^{d}	67.91±1.48^{b}	75.14±1.88^{a}	63.28±1.51^{c}	58.65±1.91^{d}	67.91±1.26^{b}
Ascorbate peroxidase	0.26±0.0097^{d}	0.32±0.0075^{b}	0.36±0.0081^{a}	0.30±0.0088^{c}	0.28±0.0077^{d}	0.32±0.0072^{b}
Catalase	111.33±4.16^{d}	131.33±3.05^{b}	144±2^{a}	122±3.46^{c}	112.66±3.05^{d}	128±3.46^{b}

★a, b, c, d, e indicates significant differences

On the basis of the above preliminary experiments, 25 and 75 mM NaCl and 100µM were identified as the appropriate concentrations. Therefore, the final phase of the experiment entailed the concurrent exposure of okra seedlings to NaCl (25 and 75 mM) and NaHS (100µM) for 7 days in a hydroponic system. To validate H_2S functionality as an abiotic stress tolerance molecule in okra, the experimental setup incorporated H_2S inhibitor PAG (100 µM) and H_2S scavenger HT (200 µM) (Table 4.6). Following the 7-day treatment period, the seedlings were analyzed for various morphological and biochemical parameters. During the growth phase in the hydroponic system, aeration was done daily, and an adequate concentration of treatments was maintained by replenishing the solution every two days.

Table 4.6. Experimental setup for investigation of NaHS efficacy as an H_2S donor to induce salt stress endurance in okra seedlings.

Control				
S1	S1N	S1NHT	S1PAG	S1NPAG
S3	S3N	S3NHT	S3PAG	S3NPAG

4.13. Evaluation of okra morphology subjected to a combination treatment in hydroponics

The study investigated the morphological responses of seedlings to various treatments involving salt (NaCl), H_2S donor (NaHS), H_2S scavenger (HT), and synthesis inhibitor (PAG) (Fig. 4.14A). The treatments were designated using varied suffixes such as S1 and S3 (indicating different salt concentrations), N (NaHS), HT (hypotaurine), and

PAG (DL-propargylglycine) (Table 4.6). Different combinations used in the study are S1, S1N, S1NHT, S1PAG, S1NPAG, S3, S3N, S3NHT, S3PAG, and S3NPAG.

Salt stress negatively impacted the seedling morphology. With an increase in NaCl concentration from S1 to S3, a significant reduction of 16% and 50% in shoot length compared to the control was evident (Fig. 4.14B). Root length measurements followed a similar pattern, with salt stress causing 26 and 63% reductions in S1 and S3 treatments compared to control okra seedlings (Fig. 4.14C). Salt stress has been documented to reduce plant height through multiple mechanisms, including ionic accumulation (Dikobe et al., 2021), elevated osmotic pressure restricting water uptake (Dikobe et al., 2021), inhibited cell division and expansion (Rahneshan et al., 2018), and increased hydrogen peroxide levels (Fig. 4.18A) (Raju and Prasad, 2021). Similar responses have been documented in other salt-stressed crops, including *C. sativum* L. (Kapoor et al., 2023) and *P. vulgaris* L. (Dawood et al., 2022a).

However, the application of NaHS demonstrated a protective effect, improving shoot elongation by 11 and 24% in S1N and S3N seedlings than S1 and S3 salt-stressed okra (Fig. 4.14B). Similarly, NaHS application improved root length in S1N and S3N seedlings by 20 and 47%, respectively. Earlier, 25 μM NaHS was reported to enhance the shoot-root length of salt-stressed *B. juncea* L. (Verma et al., 2023a). Similarly, treatment with 100 μM NaHS reportedly improved the growth parameters of *C. sativus* L. during NaCl exposure (Liu et al., 2022a). Therefore, as per available literature, NaHS mediated the mitigation of the negative effect of salinity on okra growth attributes correlated with the increased internal H_2S levels in plants (Valivand et al., 2019a; Dawood et al., 2022a; Gautam et al., 2022).

The ameliorative effect of NaHS was subsequently confirmed through the application of HT. The protective influence of NaHS was negated by HT mediated scavenging of H_2S and thus reduction of shoot length by 20 and 38% in S1NHT and S3NHT treatments than S1N and S3N was observed. Likewise, the root length of S1NHT and S3NHT okra seedlings was decreased by 26 and 52%, respectively, compared to S1N and S3N seedlings (Fig. 4.14B-C). HT has already been reported to scavenges H_2S and reverse the effect of NaHS on growth parameters in stressed plants like in case of *S. indicum* under lead stress (Amooaghaie and Enteshari, 2017), *M. hupehensis* under NaCl stress (Wei et al., 2019), *C. pepo* under nickel stress (Valivand and Amooaghaie,

2021a), *A. thaliana* under manganese stress (Hou et al., 2022), and *C. sativus* L. under NaCl stress (Liu et al., 2022a). The reversal of NaHS protective effects demonstrated that H_2S plays an essential role in plant growth regulation during stress conditions.

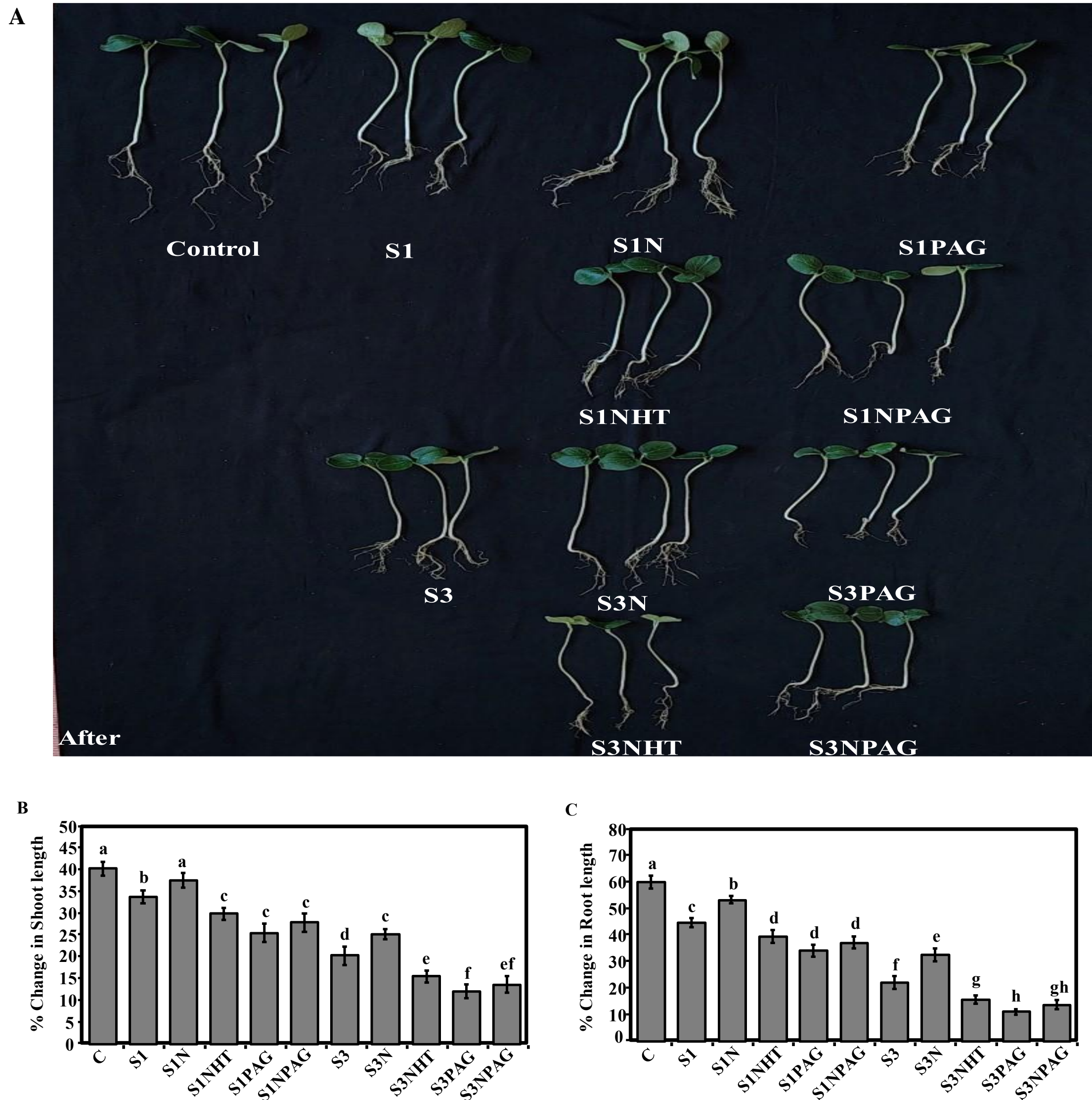

Figure 4.14. (A)The figure illustrates the efficacy of NaHS in preserving the morphological characteristics of okra subjected to salt stress. The graphs illustrate the significant improvement in (B) shoot length and (C) root length of salt-stressed okra on NaHS supplementation in the absence of H_2S scavenger and inhibitor.

Further investigation of the endogenous H_2S role in stress alleviation was assessed using inhibitor PAG. PAG exposure was found to further reduce the shoot and root length by 24-41 and 23-49%, respectively, in S1PAG-S3PAG treatments compared to

S1 and S3 treated okra seedlings (Fig. 4.14B-C). Luo et al. (2023) reported a decline in the plant height of NaCl-stressed *C. sativus* L. seedlings on PAG exposure. Similar results have been reported in several other crops, including *V. mungo* and *V. radiata* under chromium (VI) stress (Husain et al., 2022), *M. sativa* under cadmium stress (Yang et al., 2021), and *O. sativa* L. seedlings under arsenate stress (Mishra and Singh, 2021).

Notably, NaHS supplementation was not able to efficiently counteract the negative effects of salt stress in the presence of PAG. A non-significant variation in the shoot and root elongation of S1NPAG and S3NPAG with respect to S1PAG and S3PAG salt-stressed okra was noticed. So, the protective effect of NaHS against salt stress might have weakened due to the PAG-induced inhibition of endogenous H_2S synthesis.

4.14. NaHS-mediated rescue of photosynthetic pigments, biomass, and carbohydrate content of NaCl-stressed okra

The present study demonstrated that increasing NaCl concentrations, S1 to S3, significantly impaired multiple physiological parameters in seedlings, including biomass, chlorophyll, carotenoid, and carbohydrate, than the control. The photosynthetic pigment composition was notably affected, as chlorophyll a content was decreased by 9-33% in S1 and S3 treated okra seedlings, respectively, compared to the control (Fig. 4.15A). Chlorophyll b exhibited a similar trend and was reduced by 12 and 36% in S1 and S3 treated okra seedlings, respectively. Likewise, carotenoid content was diminished by 6-26% in S1 and S3 treatments compared to the control. The decrease in photosynthetic pigment is likely due to instability of the pigment–protein complex (Trifunović-Momčilov et al., 2021; Yan et al., 2022), increased chlorophyllase activity at high pH (Yan et al., 2022), increased ROS content (Fig. 4.18A; Zhong et al., 2023; Kaya et al., 2024), and slow synthesis or fast breakdown of the pigments (Rahneshan et al, 2018; Trifunović-Momčilov et al., 2021).

The content of photosynthetic pigments is directly associated with the carbohydrate and fresh biomass accumulation of plants (Zhong et al., 2023; Gautam et al., 2024). Reduced pigment concentration during stress results in a drop of photosynthetic rate, which restricts the availability of CO_2, as a result, lower the amount of carbohydrates (Hassanein et al., 2009). Therefore, fresh biomass and carbohydrate content of okra

seedlings exhibited parallel responses to pigment content and were reduced by 20-58 and 28-47%, respectively in S1-S3 okra seedlings compared to the control (Fig. 4.15D-E).

NaHS supplementation to salt-stressed seedlings in S1N and S3N demonstrated protective effects and improved chlorophyll a content by 10 and 20%, as compared to S1 and S3 seedlings (Fig. 4.15A). Likewise, chlorophyll b and carotenoid were recovered on NaHS exposure by 11-20 and 9-16%, respectively, in S1N and S3N seedlings (Fig. 4.15B-C). Earlier reports suggested that H_2S-induced pigment protection due to enhanced chlorophyll production and protection of cellular structures from ROS damage (Younis and Mansour, 2024). Reports further document that H_2S improved the stomatal aperture, which increased the availability of CO_2 in mesophyll cells, thereby improving the photosynthetic performance (Younis and Mansour, 2024). Wei et al. (2021b) reported that H_2S enhances the synthesis of chlorophyll precursor (porphyrin IX) thereby increasing pigment content. Likewise, on NaHS supplementation, fresh biomass and carbohydrate were restored in salt-exposed seedlings by 18-44 and 36-24%, respectively (Fig. 4.15D-E). The enrichment of carbohydrate and fresh biomass by exogenous supplementation of H_2S could be attributed to the H_2S-induced improvement in pigment content. Previous reports have demonstrated that H_2S, administered as NaHS, effectively restored carbohydrate levels in various plant species, including *C. sativus* L. (Liu et al., 2022a) and *Dendranthema morifolium* Ramat. (Wei et al., 2021a). Kaya et al. (2024) have earlier reported that NaHS treatment enhanced pigment content and photosynthetic efficiency in salt-stressed *C. annuum* L., which ultimately improved growth.

The protective role of NaHS in pigment content accumulation and its associated parameters was further validated through HT application. The chlorophyll a and chlorophyll b content were reduced by 18-28% in S1NHT and S3NHT seedlings, with respect to S1N and S3N, respectively (Fig. 4.15A-B). Similarly, carotenoid content was reduced by 17 and 24% in S1NHT and S3NHT seedlings, respectively (Fig. 4.15C). HT exposure likewise, negated the protective effects of NaHS on fresh weight and carbohydrate content, resulting in a reduction of 28-45 and 38-39%, respectively in S1NHT-S3NHT seedlings compared to S1N-S3N seedlings (Fig. 4.15D-E). HT-induced inhibition of photosynthetic pigment and its associated parameters is attributed

to the scavenging of H_2S by HT, which restricts the internal functioning of H_2S, as evidenced by the reduced H_2S content (Fig. 4.22). The results are consistent with the earlier reports where HT has been reported to revoke the impact of H_2S on the pigment content in stressed *A. thaliana* (Hou et al., 2022), *Eruca sativa* Mill. (Khan et al., 2018), and blueberry (Tang et al., 2020). Likewise, carbohydrate and fresh biomass were also reduced by HT in *C. sativus* L. under NaCl stress (Liu et al., 2022a), *O. sativa* L. under high temperature stress (Gautam et al., 2022), *O. sativa* L. under cadmium stress (Mostofa et al., 2015b), and *C. pepo* under nickel stress (Valivand and Amooaghaie, 2021a).

Moreover, PAG exposure reduced the pigment, carbohydrate, and fresh biomass in okra seedlings. It caused a reduction of 18-30% in chlorophyll a, 22-32% in chlorophyll b, and 20-29% in carotenoids in S1PAG-S3PAG seedlings, respectively, compared to S1-S3 seedlings (Fig. 4.15A-C). PAG treatment to salt-stressed S1 and S3 seedlings similarly decreased fresh biomass and carbohydrate accumulation. The fresh biomass was reduced by 31 and 46% in S1PAG and S3PAG seedlings, respectively, compared to S1 and S3 seedlings (Fig. 4.15D). Likewise, carbohydrate content was reduced by 34 and 53% in S1PAG and S3PAG seedlings, respectively, relative to S1 and S3 seedlings (Fig. 4.15E).

Notably, NaHS supplementation significantly counteracted the PAG-induced reductions and improved pigment accumulation by 5-11% for chlorophyll a, 8-13% for chlorophyll b, and 9-19% for carotenoids in S1NPAG and S3NPAG treatments as compared to S1PAG and S3PAG seedlings, respectively (Fig. 4.15A-C). Likewise, the combined negative effect of PAG and NaCl on fresh biomass and carbohydrate accumulation in okra seedlings was mitigated by NaHS supplementation. The fresh biomass and carbohydrate content were enhanced by 10-34 and 14-37%, respectively, in S1NPAG-S3NPAG seedlings compared to S1PAG and S3PAG seedlings (Fig. 4.15D-E). This indicated that the stress mitigation potential of H_2S on salt-stressed okra seedlings was weakened in the presence of H_2S synthesis inhibitor PAG, but was alleviated by NaHS exposure. In an earlier study PAG has been reported to reverse the impact of H_2S on pigment, carbohydrate and fresh biomass accumulation of *O. sativa* L. under arsenate stress (Mishra and Singh, 2021). This increased sensitivity to stress

factors further emphasized the crucial protective role that H_2S plays in maintaining plant health under adverse conditions.

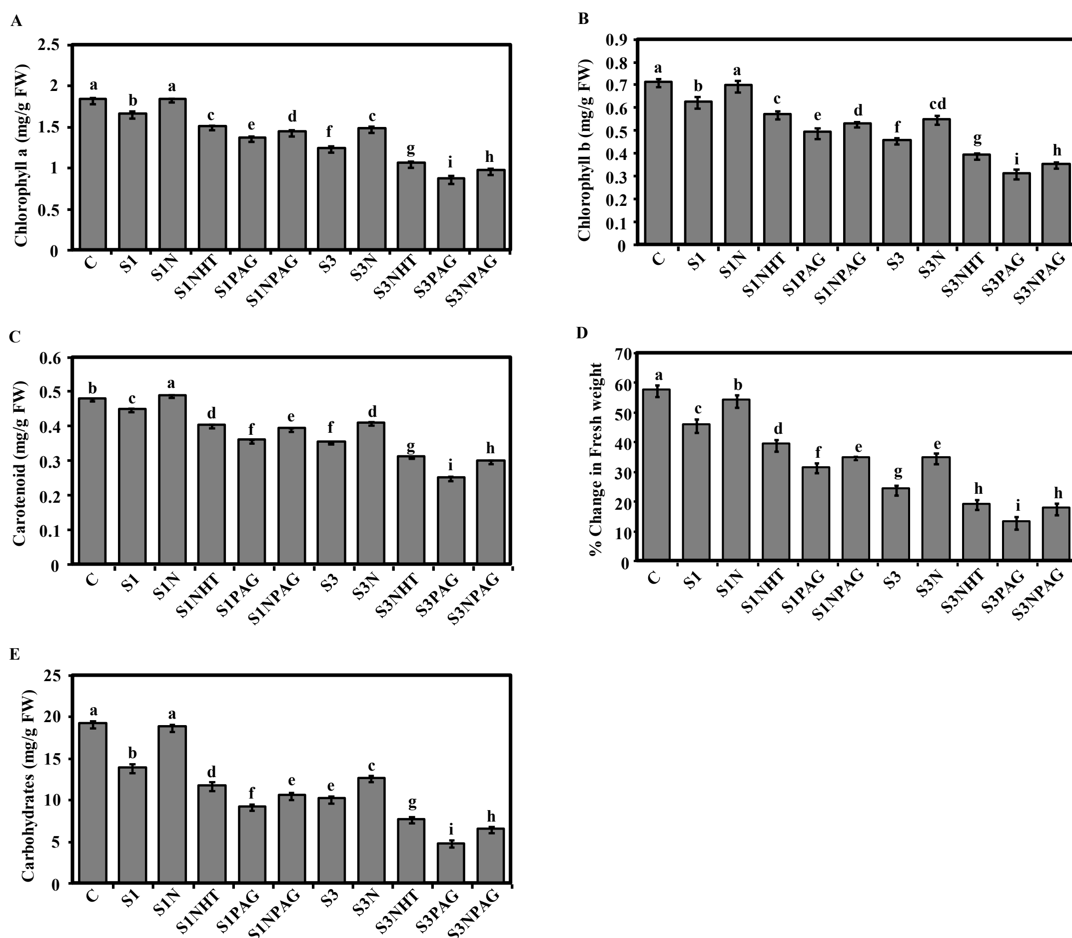

Figure 4.15. Impact of NaCl, NaHS, HT, and PAG on the photosynthetic attributes of okra. The bar graphs illustrate the significant improvement in the levels of (A) chlorophyll a, (B) chlorophyll b, (C) carotenoids, (D) fresh biomass, and (E) carbohydrates in salt-stressed okra on NaHS supplementation.

4.15. Polyphenol, flavonoid, and antioxidant responses of okra seedlings to NaCl and NaHS treatment

The study investigated the impact of salt stress and H_2S on antioxidant components of okra seedlings (Fig. 4.16A-C). Salt stress induced a significant increase of 20 and 61% in total phenolic content of S1 and S3 treated okra compared to the control (Fig. 4.16A). Similar trends were observed in flavonoid content, with an increase of 23 and 55% under salt stress in S1 and S3 treated okra seedlings. Antioxidant potential demonstrated

parallel responses, showing an increase of 7 and 28% in S1 and S3 seedlings compared to the control (Fig. 4.16C). The non-enzymatic antioxidants are also upregulated in plants in response to stress to generate an inbuilt stress-tolerant response (Gill and Tuteja, 2010). Several crops have shown comparable outcomes, including salt-stressed *T. aestivum L.* (Sadak et al., 2023), and *M. oleifera* Lam. (Azeem et al., 2023).

NaHS supplementation further enhanced the total polyphenolic and flavonoid content by 25-21 and 18%, respectively, in S1N-S3N treated seedlings than S1 and S3 stressed okra (Fig. 4.16A-B). Likewise, okra seedlings further depicted an enhancement of 17-21% in the antioxidant potential upon NaHS supplementation in S1N-S3N seedlings, respectively, than S1 and S3 seedlings. Similarly, in stressed *C. pepo* and *S. lycopersicum* L. seedlings, NaHS supplementation enriched the non-enzymatic antioxidants to regulate oxidative stress (Valivand and Amooaghaie, 2021a; Zhang et al., 2023d). Likewise, under NaCl stress, 20 and 50μM of NaHS were reported to enrich the phenolic content in *T. aestivum* L. (Alamer, 2023).

The protective role of H_2S was further confirmed through HT and PAG applications, which reversed the beneficial effects of H_2S on total phenolic, flavonoid content, and antioxidant potential of treated okra seedlings (Fig. 4.16A-C). A decline of 7% in the polyphenolic content of S1NHT and S3NHT okra seedlings than S1N and S3N okra seedlings was observed. Similarly, in S1NHT and S3NHT seedlings, flavonoid and antioxidant potential were declined by 5-6 and 5-7%, respectively, compared to the corresponding S1N and S3N treated seedlings (Fig. 4.16B-C). The application of PAG similarly resulted in a substantial decrease across all measured parameters, reducing polyphenolic content by 4-7%, flavonoids by 5%, and antioxidant potential by 3-4% in S1PAG-S3PAG treatments, respectively, compared to the corresponding S1 and S3 treated seedlings (Fig. 4.16A-C).

However, subsequent NaHS supplementation weakly negated these PAG-induced reductions on polyphenolic content, flavonoid levels, and antioxidant potential in S1NPAG and S3NPAG treated seedlings compared to their respective PAG-treated counterparts (Fig. 4.16A-C). The polyphenolic content in S1NPAG and S3NPAG seedlings was increased by 4% as compared to S1PAG and S3PAG, respectively (Fig. 4.16A). Likewise, the flavonoid content was increased by 3 and 4% in S1NPAG and S3NPAG compared to S1PAG and S3PAG okra seedlings. Similar to polyphenolic and

flavonoid content, the antioxidant potential of S1NPAG and S3NPAG seedlings was non-significantly increased by 2 and 1%, respectively, compared to S1PAG and S3PAG seedlings (Fig. 4.16C).

The effect of H_2S in modulating the non-enzymatic antioxidants and antioxidant potential was reversed by H_2S scavenger and inhibitor. These findings thus demonstrate the significant role of H_2S in modulating antioxidant responses under salt stress conditions in okra seedlings.

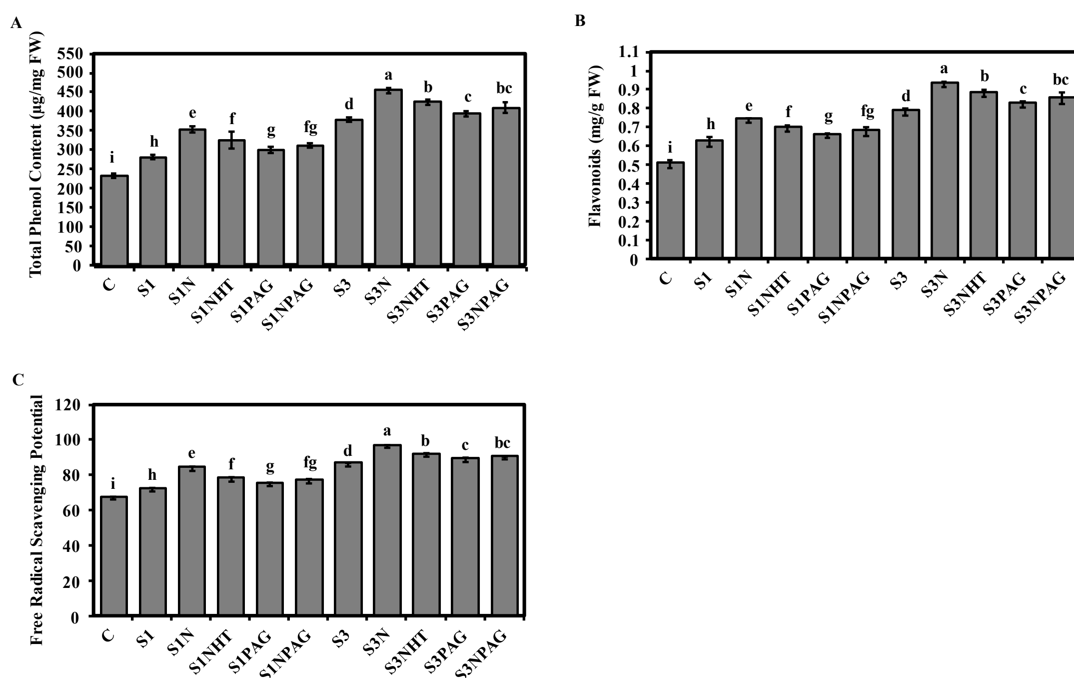

Figure 4.16. Graphs illustrate considerable changes in the (A) total phenols and (B) flavonoid accumulation in NaHS, salt, HT, and PAG-treated okra, resulting in an (C) increased antioxidant potential due to NaHS supplementation.

4.16. Enzymatic antioxidant responses of Okra

Plants possess a sophisticated antioxidant defense system to counteract ROS damage. To assess how effectively this system functions under salt stress conditions and in the presence of H_2S, we evaluated the activities of several key antioxidant enzymes, including superoxide dismutase, ascorbate peroxidase, and catalase in this study (Fig. 4.17A-C). The study revealed significant modulation in the enzymatic antioxidant system of salt-stressed okra seedlings. Superoxide dismutase activity showed a substantial increase of 27-54% in S1 and S3 salt-stressed okra seedlings compared to

the control (Fig. 4.17A). Ascorbate peroxidase demonstrated similar response patterns, and was upregulated on salt stress exposure by 30 and 71% in S1 and S3 treated seedlings compared to control (Fig. 4.17B). Catalase also exhibited a similar response pattern and was enhanced by 40-88% in S1-S3 treated okra seedlings than the control (Fig. 4.17C). The enhancement in antioxidant enzyme activities under stress is generally attributed to the inherent ability of plants to generate the stress tolerance response (Gill and Tuteja, 2010). Similar outcomes have been reported in several other plants, including *H. annuus* L. and *T. aestivum* L. under NaCl stress (Younis and Mansour, 2023; Alamer, 2023). However, this system becomes incompetent under conditions of extreme stress, leading to reduced growth and development.

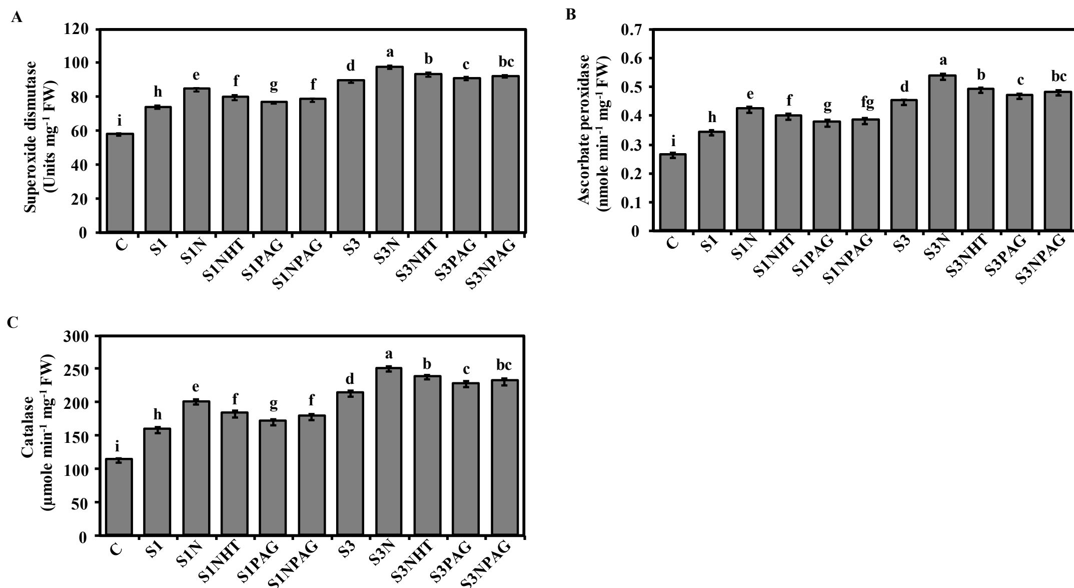

Figure 4.17. Impact of NaCl, NaHS, HT, and PAG on the activities of (A) superoxide dismutase, (B) ascorbate peroxidase, and (C) catalase. NaHS was observed to augment the activities of superoxide dismutase, catalase, and ascorbate peroxidase, hence promoting increased resilience to salt stress in okra.

NaHS supplementation to salt-stressed okra seedlings significantly enhanced antioxidant enzyme activities compared to their respective salt treatments, S1 and S3. Superoxide dismutase, ascorbate peroxidase, and catalase activity were upregulated by 9-15, 19-23, and 17-26%, respectively, compared to S1-S3 seedlings (Fig. 4.17A-C). H_2S has been earlier reported to regulate oxidative stress responses by enriching antioxidant enzyme activities in *O. sativa*, *C. sativus*, and *B. juncea* L. (Mostofa et al., 2015b; Qi et al., 2019; Liu et al., 2022a; Kaur et al., 2022).

The regulatory role of H_2S on enzymatic antioxidants was confirmed through HT treatment, which decreased antioxidant enzyme activities in salt-stressed okra seedlings. HT exposure reduced superoxide dismutase activity by 4-6% and ascorbate peroxidase by 6-8% in S1NHT-S3NHT okra seedlings, compared to S1N and S3N seedlings (Fig. 4.17A-B). Similarly, catalase activity was decreased by 5-9% in S1NHT-S3NHT treated okra relative to S1N and S3N seedlings (Fig. 4.17C). Similar outcomes have been observed in *A. thaliana* under manganese stress (Hou et al., 2022), *S. alterniflora* and *C. malaccensis* under salt stress (Li et al., 2020b).

Similarly, the regulatory role of endogenous H_2S on enzymatic antioxidants was confirmed through PAG exposure to salt-stressed okra seedlings. In S1PAG and S3PAG okra seedlings, superoxide dismutase activity resulted in a significant increase of 2-4% than S1-S3 seedlings (Fig. 4.17A). Similarly, on PAG exposure to salt-stressed okra seedlings, ascorbate peroxidase and catalase activity were also enhanced significantly by 4-10% and 6-8%, respectively, in S1PAG-S3PAG seedlings, then their corresponding salt-treated okra seedlings (Fig. 4.17B-C). Similar outcomes have been observed in *Zea mays* L. and *M. sativa* under heat and cadmium stress (Ye et al., 2020; Yang et al., 2021).

Subsequent co-application of NaHS to PAG-treated salt-stressed okra seedlings was not able to significantly affect antioxidant enzyme activities. Superoxide dismutase activity was increased by only 1-2% in S1NPAG-S3NPAG seedlings compared to S1PAG and S3PAG treatments, respectively (Fig. 4.17A). Similarly, ascorbate peroxidase activity was non-significantly enhanced by 2% in both S1NPAG and S3NPAG compared to their respective PAG-treated counterparts (Fig. 4.17B). Likewise, catalase activity showed a non-significant change of 2-4% in S1NPAG-S3NPAG seedlings relative to the corresponding S1PAG and S3PAG treatments (Fig. 4.17C).

These findings demonstrate that a continuous and robust H_2S is required to modulate enzymatic antioxidant responses against salt-induced oxidative stress in okra, and the protective effect of H_2S on okra gets reversed by exposure to H_2S scavenger (HT) and synthesis inhibitor (PAG).

4.17. Oxidative stress parameters were regulated in NaCl-stressed okra on NaHS treatment

According to Hasanuzzaman et al. (2021), one of the most prominent detrimental impacts of salinity on plants is oxidative stress, which is linked to the substantial production of ROS. Therefore, this study investigated oxidative stress accumulation in okra seedlings by examining hydrogen peroxide and malondialdehyde accumulation under various treatments (Fig. 4.18A-B). Salt stress induced a substantial increase in hydrogen peroxide content, with elevations of 97 and 241% observed in S1 and S3 treated okra seedlings compared to the control (Fig. 4.18A). Lipid peroxidation, measured as malondialdehyde content, showed parallel responses to hydrogen peroxide accumulation (Fig. 4.18B). On salt exposure to okra seedlings, malondialdehyde level was increased by 79 and 181% in S1 and S3 treated okra seedlings compared to the control (Fig. 4.18B). An abrupt rise in hydrogen peroxide and malondialdehyde levels was earlier reported in *L. esculentum* Mill., *O. sativa* L., and *C. sativus* L. under NaCl stress (Khan et al., 2021; Wei et al., 2021b; Liu et al., 2022a). Under salt stress, the copper and iron ion availability promoted Fenton reactions that produce excess hydroxyl radicals, ultimately compromising overall plant function and health (Singh and Prasad, 2015). Besides, an elevated level of lipid peroxidation is attributed to the increased production of ROS (Yin et al., 2016; Abdelrhim et al., 2021).

NaHS supplementation effectively mitigated the oxidative stress, and reduced hydrogen peroxide and malondialdehyde levels by 21-31 and 21-30%, respectively, in S1N and S3N treatments compared to their corresponding salt-stressed seedlings (Fig. 4.18A-B). The mitigation of oxidative stress was attributed to improved antioxidant potential of okra seedlings (Fig. 4.16C), which was manifested through elevated antioxidant enzyme activities and increased accumulation of non-enzymatic antioxidants following NaHS administration to salt-stressed okra seedlings. Besides, H_2S, being a reducing agent, may directly scavenge ROS (He et al., 2018). Similar results have been reported in other seedlings where H_2S exposure minimizes oxidative stress, including *T. aestivum* L. and *O. sativa* L. (Ding et al., 2019; Wei et al., 2021b).

The protective role of H_2S on oxidative stress was further validated through HT and PAG treatments. HT exposure dramatically increased hydrogen peroxide content by 69 and 43% in S1NHT and S3NHT treatments compared to NaHS-treated salt seedlings

(Fig. 4.18A). Likewise, malondialdehyde content was elevated on HT exposure by 41-66% in S1NHT-S3NHT treated okra compared to S1N and S3N seedlings, respectively (Fig. 4.18B). Similarly, PAG treatment enhanced hydrogen peroxide and malondialdehyde accumulation by 30-49 and 19-33% in S1PAG-S3PAG treatments relative to S1 and S3 treated salt-stressed okra seedlings (Fig. 4.18A-B). Further, supplementation of S1PAG and S3PAG seedlings with NaHS minimized the inhibitory effect of PAG and reduced the hydrogen peroxide and malondialdehyde content by 6-10 and 3-4%, respectively, in S1NPAG-S3NPAG treated okra seedlings (Fig. 4.18A-B). These observations demonstrated the protective role of H_2S against oxidative damage under salt stress conditions (Fig. 4.18B).

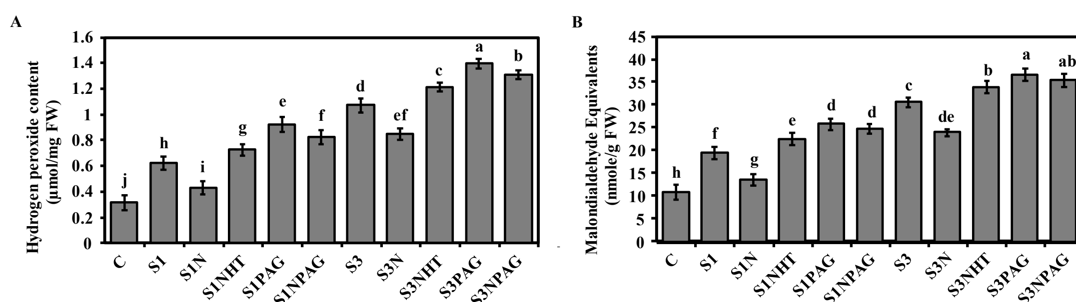

Figure 4.18. The bar graphs illustrate the effect of NaHS supplementation on oxidative stress markers (A) Hydrogen peroxide, and (B) Malondialdehyde, indicative of lipid peroxidation. NaHS exposure considerably modulated the amount of hydrogen peroxide and malondialdehyde, indicating its potential involvement in enhancing salt stress tolerance in okra.

The exacerbation of oxidative stress by blocking H_2S production using PAG or removing it from the system via HT corresponds with the reduced antioxidant potential of plants due to lowered activity of enzymatic and non-enzymatic antioxidants (Fig.4.16-4.17). In earlier studies, HT elevated oxidative stress in *A. thaliana*, *C. sativus* L., *Z. mays* L., *S. alterniflora,* and *C. malaccensis* (Li et al., 2020b; Ye et al., 2020; Hou et al., 2022; Liu et al., 2022a). Similarly, PAG was reported to induce oxidative stress in *V. mungo* and *V. radiata* under chromium (VI) stress (Husain et al., 2022), *S. alterniflora* and *C. malaccensis* under NaCl stress (Li et al., 2020b), and *O. sativa* L. under ammonia stress (Guo et al., 2017).

It is concluded from the above findings that H_2S facilitated salt stress tolerance in okra seedlings through maintenance of cellular homeostasis via enhancement of antioxidant

capacity, whereas its absence resulted in susceptibility to stress-induced damage. The experimental data suggested that H_2S functions as a critical signalling molecule that regulates oxidative stress responses and confers protection to okra seedlings against NaCl-induced cellular deterioration.

4.18. Altered protein content in okra

This study examined protein content modifications in okra seedlings under various treatments. Salt stress significantly impaired protein accumulation, causing reductions of 25-48% in S1 and S3 treatments compared to control (Fig. 4.19). Enhanced protein oxidation has been reported in *S. bicolor* L. and *T. aestivum* L. under salt stress due to the increased production of ROS (Yin et al., 2016; Abdelrhim et al., 2021).

However, NaHS supplementation demonstrated a protective effect and enhanced protein levels by 22-35% in S1N and S3N treatments, respectively (Fig. 4.19). Previous reports have demonstrated that H_2S, administered as NaHS, effectively restored protein levels in various plant species, including *C. sativus* L. (Liu et al., 2022a) and *D. morifolium* Ramat. (Wei et al., 2021a). H_2S achieves this protein restoration through multiple mechanisms, specifically by enhancing protein synthesis, ensuring correct protein folding, and facilitating protein transport processes (Wei et al., 2021b).

The regulatory role of H_2S on protein content was further validated through HT application, which reversed the beneficial effects of NaHS treatment and reduced protein content by 34-43% in S1NHT-S3NHT treatments compared to S1N and S3N seedlings, respectively (Fig. 4.19). HT has earlier been reported to reverse the effect of H_2S on protein content in salt-stressed *S. melongena* and S. *Lycopersicon* (Raju and Prasad, 2021).

Similarly, PAG treatment decreased the protein levels by 38 and 54% in S1PAG and S3PAG treatments, respectively, compared to salt-stressed okra seedlings (Fig. 4.19). Likewise, PAG has earlier been reported to reverse the impact of H_2S on protein content in *O. sativa* L. under arsenate stress (Mishra and Singh, 2021). Notably, subsequent NaHS supplementation improved PAG-induced reductions and significantly increased the protein level by 19-39% in S1NPAG-S3NPAG treated okra seedlings, respectively (Fig. 4.19). These findings demonstrate the significant role of H_2S in maintaining protein homeostasis under salt stress conditions in okra seedlings.

Protein content is also associated with nitrogen metabolism (Farhan et al., 2024). Therefore, the impaired GS-GOGAT pathway under salinity could also have induced a decline in the accumulation of protein content in salt-stressed okra seedlings (Debouba et al., 2006; Meng et al., 2016).

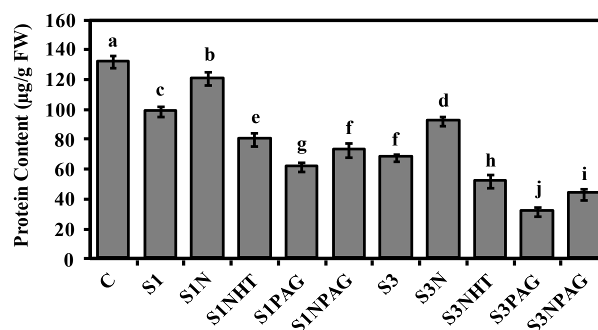

Figure 4.19. The illustration depicts the impact of NaHS exposure on protein accumulation in salt-stressed okra. NaCl exposure decreased the protein content of okra, whereas concurrent NaHS exposure significantly enhanced it. The simultaneous exposure of scavenger HT, inhibitor PAG, and NaHS indicated a probable function of H_2S in the observed alterations of protein build-up in okra.

4.19. Regulation of nitrogen metabolism by NaHS in NaCl-stressed okra seedlings

The present study investigated the impact of various treatments on nitrogen metabolism in okra, addressing the well-documented disruptive effects of salt stress on nitrogen metabolism-related processes. Previous research by Liu et al. (2022c) and Tzortzakis et al. (2022) has established that salt stress can significantly impair nitrogen metabolism in plants, particularly affecting nitrification and ammonification processes, thereby compromising nitrogen uptake and assimilation. The investigation specifically examined the effects of NaCl, NaHS, HT, and PAG on nitrogen metabolism and its associated enzymatic activities, aiming to elucidate the regulatory mechanism involved in nitrogen homeostasis under salt stress conditions. This comprehensive approach provided insights into the interplay between salt stress and H_2S signalling in modulating plant nitrogen metabolism.

4.19.1. Analysis of inorganic nitrogen content in salt-stressed okra seedlings on varied exposure

The study examined the effects of various treatments on inorganic nitrogen content in okra seedlings (Fig. 4.20A-C). Salt stress significantly impaired nitrate content, and reduced it by 27 and 59% in S1 and S3 treated okra seedlings, respectively, relative to the control (Fig. 4.20A). Nitrite accumulation demonstrated similar response patterns. Salt stress reduced nitrite content by 23 and 54% in S1 and S3 treated okra relative to the control (Fig. 4.20B). The observed decrease in nitrate and nitrite content can be due to the downregulation of nitrate transporter (*NRT1*;1), competitive inhibition of nitrate uptake by NaCl (Ashraf et al., 2018; Parihar et al., 2021), or deterioration of root membrane (Debouba et al., 2006). The decreased levels of nitrate and reduced nitrate reductase enzyme activity under stress result in lower nitrite production, since nitrate reductase is responsible for converting nitrate to nitrite (Manai et al., 2014).

NaHS supplementation effectively counteracted NaCl-induced impairment, and restored nitrate and nitrite levels by 25-53 and 20-42%, respectively, in S1N and S3N treated okra seedlings compared to S1 and S3 seedlings (Fig. 4.20A-B). The increased nitrate level by H_2S is due to improved uptake and transportation of nitrate within the plant system (Rizwan et al., 2019; Valivand and Amooaghaie, 2021b) and reduced oxidative stress (Fig. 4.18).

HT exposure reversed the protective effect induced by NaHS exposure in salt-stressed okra seedlings and reduced nitrate and nitrite content by 29-50 and 28-48%, respectively, in S1NHT-S3NHT seedlings compared to S1N and S3N seedlings (Fig. 4.20A-B). A similar outcome has been observed in *C. annuum* under drought stress (Kaya and Shabala, 2023). PAG treatment, on the other hand, decreased nitrate level by 20-44% and nitrite level by 23-46% in S1PAG and S3PAG seedlings relative to salt-treated S1 and S3 okra seedlings (Fig. 4.20A-B). Notably, subsequent NaHS supplementation to PAG and salt-stressed seedlings restored nitrate and nitrite content by 7-19 and 7-16%, respectively, in S1NPAG and S3NPAG okra seedlings (Fig. 4.20A-B).

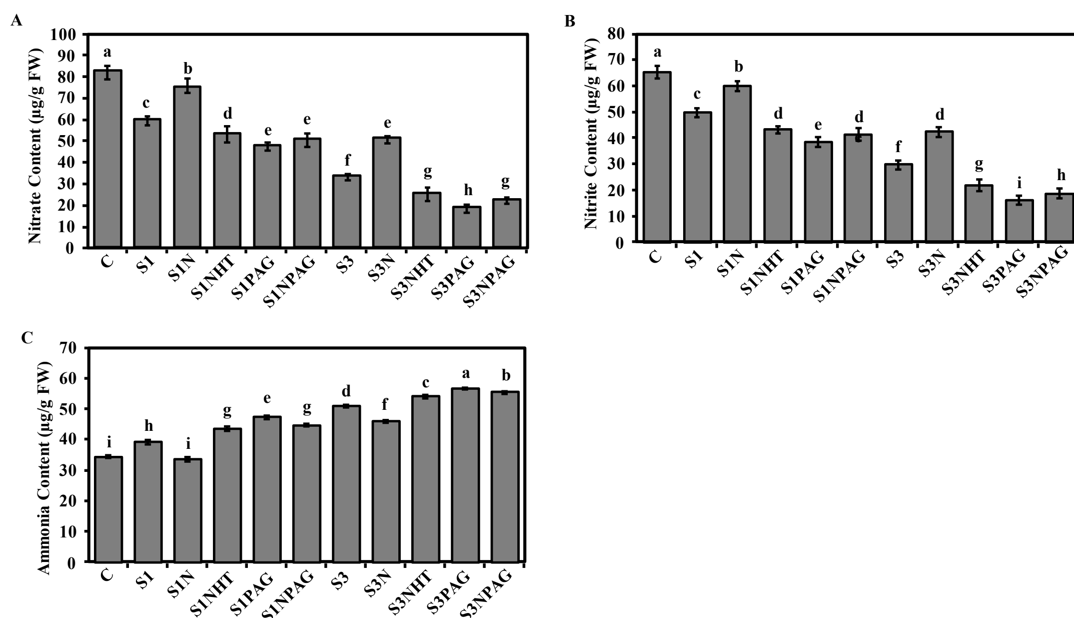

Figure 4.20. Impact of NaCl, NaHS, HT, and PAG on the inorganic nitrogen content. The bar graphs illustrate the significant increase in the levels of (A) nitrate and (B) nitrite in salt-stressed okra following exposure to NaHS. NaHS supplementation was observed to decrease the (C) ammonia levels of salt-stressed okra, suggesting enhanced stress tolerance.

Ammonia accumulation, however, exhibited contrasting responses. Salt stress increased ammonia content by 14 and 48% in S1 and S3 treatments, respectively, compared to the control (Fig. 4.20C). The increase in ammonia level under salt stress is due to decreased nitrate reductase and nitrite reductase activity under salinity, which prevented the GS-GOGAT pathway from delivering ammonia to the amino acid synthesis pathway, thereby impeding ammonia assimilation (Debouba et al., 2006; Meng et al., 2016). The decreased ammonia assimilation leads to increased ammonia levels and decreased protein content under NaCl stress in okra seedlings. On the other hand, under salt stress enhanced proteolysis has also been observed that leads to increased ammonia content (Fig. 4.19; Wang et al., 2007). Ammonia accumulation induces various cellular disruptions (Xiao et al., 2023) that could be the reason for the reduced plant growth of okra under NaCl stress (Fig. 4.14).

NaHS supplementation reduced the elevated ammonia levels by 14 and 10% in S1N and S3N treated seedlings relative to S1 and S3 seedlings (Fig. 4.20C). The reduction in ammonia level by H_2S supplementation to NaCl-toxicated okra seedlings could be

attributed to reduced proteolysis (Fig.4.19) and improved GS-GOGAT performance as documented earlier in *O. sativa* L. and *C. annuum* (Fig. 4.21C-D; Rizwan et al., 2019; Kaya and Shabala, 2023). This is due to improved content of H_2S in these seedlings and its associated signaling. Furthermore, the decreased ammonia content by NaHS in NaCl stressed okra seedlings indicated effective ammonia incorporation into glutamate, supported by enhanced growth and protein content (Fig.4.14 and 4.19).

HT exposure, however, enhanced ammonia accumulation in S1NHT-S3NHT by 29-18% compared to S1N and S3N treated seedlings, respectively (Fig. 4.20C). A similar outcome has been observed in *C. annuum* under drought stress (Kaya and Shabala, 2023). Likewise, PAG treatment increased ammonia content in S1PAG and S3PAG seedlings than S1 and S3 treated seedlings by 21 and 11%, respectively. However, subsequent NaHS application reduced these elevated ammonia levels by 6 and 2% in S1NPAG and S3NPAG, respectively, compared to their corresponding S1PAG-S3PAG seedlings (Fig. 4.20C). Raju and Prasad (2023) observed enhanced ammonia accumulation under PAG exposure to salt-stressed *S. melongena* and *S. Lycopersicon*. The raised ammonia level observed in SNHT, SPAG, and SNPAG treatment provided additional evidence for the endogenous H_2S regulatory function. This finding demonstrated the crucial role of endogenous H_2S in ammonia to glutamate assimilation, which explained the enhanced growth patterns observed in the study. Overall, these findings demonstrate the complex regulatory role of H_2S in modulating inorganic nitrogen metabolism under salt stress conditions, with distinct patterns observed across different nitrogen forms.

4.19.2. Analysis of nitrogen metabolism enzymes in salt-stressed okra seedlings on varied exposure.

The study investigated the effects of H_2S on key nitrogen metabolism enzymes, including nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, and glutamate dehydrogenase in okra seedlings under salt stress conditions through varied treatments, including its scavengers and inhibitors. These enzymes showed varied expression in the treatments under study (Fig. 4.21A-E).

Salt stress significantly inhibited nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase activities, with more pronounced effects at higher

NaCl concentrations (Fig. 4.21A-D). Nitrate reductase and nitrite reductase activity decreased in S1-S3 treated seedlings by 21-49 and 18-45%, respectively, compared to the control okra seedlings (Fig. 4.21A-B). The salinity-induced decline in nitrate reductase activity has been reported for *S. lycopersicum* L. (Debouba et al., 2006), *V. radiata* (Ullah et al., 2023), and *B. juncea* L. (Sami et al., 2021). Similarly, nitrite reductase activity was reported to be inhibited under stress in *C. annuum*, *V. faba* L., and *O. sativa* L., which corroborates our findings (Siddiqui et al., 2021; Rizwan et al., 2022; Kaya and Shabala, 2023). The reduction in the activities of nitrate reductase and nitrite reductase under stress occurs due to multiple reasons, including decreased nitrate uptake, downregulation of OsNiR expression, and enhanced hydrogen peroxide production (Rizwan et al., 2019). Furthermore, the halted nitrate reductase and nitrite reductase activity under salinity prevents the GS-GOGAT pathway from delivering ammonia to the amino acid synthesis pathway, thereby impeding nitrogen metabolism (Debouba et al., 2006; Meng et al., 2016).

On NaHS exposure, the nitrate reductase activity was upregulated by 23 and 30% in S1N and S3N seedlings compared to S1 and S3 treated okra (Fig. 4.21A). Likewise, nitrite reductase activity was upregulated by 24 and 35% in S1N and S3N treated okra seedlings compared to S1 and S3 seedlings (Fig. 4.21B). Previous research has reported that NaHS supplementation enhanced nitrate reductase activity in several plant species under salt stress conditions. This effect has already been documented in *B. platyphylla*, *C. paliurus*, and *P. vulgaris* L. (Ma et al. 2019; Chen et al. 2021b; Dawood et al., 2022a).

On HT exposure, the activities of nitrate reductase and nitrite reductase in S1NHT-S3NHT were diminished by 30-37 and 25-31%, respectively, compared to S1N and S3N treated okra seedlings (Fig. 4.21A-B). A similar outcome has been observed in *L. esculentum* Mill. under salt stress and *C. annuum* under drought stress (Khan and AlZuaibr, 2022; Kaya and Shabala, 2023). Similarly, PAG exposure to S1PAG and S3PAG declined the nitrate reductase and nitrite reductase activity by 26-31 and 16-15%, respectively, compared to S1 and S3 seedlings (Fig. 4.21A-B). A similar outcome was noticed in *S. melongena* and *S. Lycopersicon*. under salt stress (Raju and Prasad, 2023).

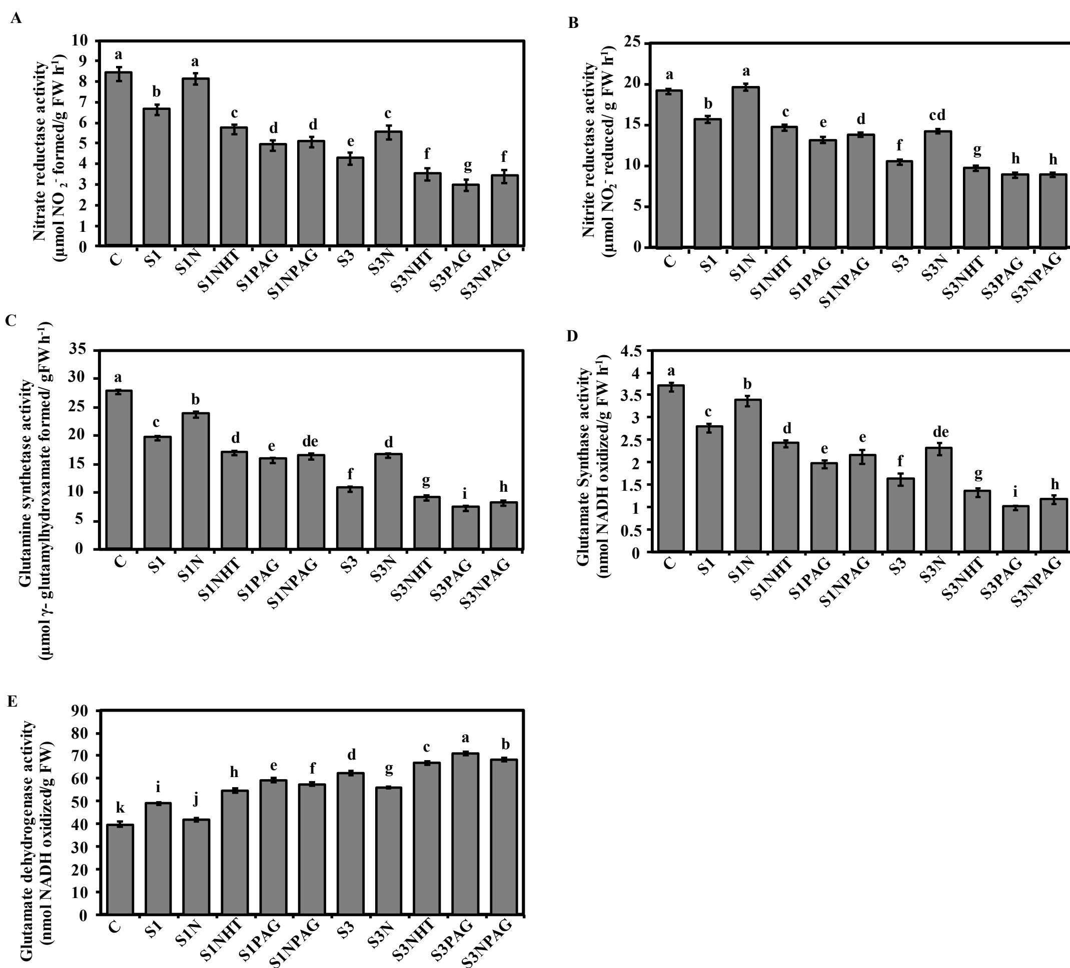

Figure 4.21. Impact of NaCl, NaHS, HT, and PAG on nitrogen metabolism enzymes. The graphs illustrate the NaHS-mediated enhancement of activity in (A) nitrate reductase, (B) nitrite reductase, (C) glutamine synthetase, and (D) glutamate synthase in salt-stressed okra, signifying the rescue of nitrogen metabolism under stress circumstances by NaHS. The activity of (E) glutamate dehydrogenase was significantly diminished upon exposure to NaHS, suggesting its possible involvement in the stress control of okra.

NaHS application to PAG-supplemented salt-stressed okra seedlings weakly recovered the PAG-induced reduction in the nitrate reductase and nitrite reductase activity of salt-treated okra seedlings. In S1NPAG and S3NPAG seedlings, nitrate reductase activity was improved by 3 and 15% compared to S1PAG and S3PAG seedlings, respectively (Fig. 4.21A). Likewise, nitrite reductase activity of S1NPAG and S3NPAG was restored by 5 and 1%, respectively, compared to S1PAG and S3PAG (Fig. 4.21B). The role of H_2S in improving the activity of nitrate reductase and nitrite reductase under salt

stress in okra seedlings is affirmed by the use of PAG and HT, which inhibited endogenous H_2S synthesis or scavenged H_2S, leading to a decline in their activity.

Ammonia-assimilating enzymes glutamine synthetase, glutamate synthase, and glutamate dehydrogenase are crucial for plant development. The GS-GOGAT pathway efficiently assimilates ammonia in plants through sequential enzyme action, glutamine synthetase first incorporates ammonia into glutamine, which is subsequently converted by glutamate synthase to glutamate (Lea and Ireland, 1999). Under stress conditions, the GS-GOGAT pathway becomes compromised, with both glutamine synthetase and glutamate synthase showing reduced enzymatic activity, similar to the observed decline in nitrate and nitrite reductase activity.

In the present study, glutamine synthetase activity was inhibited by 29-61%, while glutamate synthase was reduced by 25-56% in S1-S3 treated okra seedlings, respectively, compared to control seedlings (Fig. 4.21C-D). These findings align with reported inhibition of glutamine synthetase and glutamate synthase activity in *L. esculentum* Mill. and *V. radiata* L. under NaCl stress (Khan and AlZuaibr, 2022; Ullah et al., 2023). The decreased activity of glutamine synthetase and glutamate synthase can be attributed to their oxygen-labile nature and the elevated production of ROS under NaCl stress, as previously demonstrated by Raju and Prasad (2023).

NaHS treatment effectively counteracted these inhibitory effects and recovered the activities of glutamine synthetase and glutamate synthase enzymes (Fig. 4.21C-D). The glutamine synthetase activity was improved by 21-54% in S1N and S3N seedlings compared to the S1 and S3 seedlings, respectively (Fig. 4.21C). Likewise, the activity of glutamate synthase enzyme was improved by 22-43% in S1N and S3N seedlings compared to salt-stressed okra seedlings (Fig. 4.21D). H_2S supplementation to stressed seedlings was earlier found to improve performance of the GS-GOGAT pathway in *O. sativa* L. and *C. annuum* seedlings under nickel and drought stress, thereby effectively regulating ammonia levels (Rizwan et al., 2019; Kaya and Shabala, 2023).

However, when HT was administered to NaHS-treated salt-stressed seedlings, it reversed the beneficial effects of NaHS, resulting in decreased enzyme activities (Fig. 4.21C-D). Glutamine synthetase was reduced by 28-45%, while glutamate synthase was reduced by 28-42% in S1NHT and S3NHT seedlings, respectively, compared to S1N and S3N seedlings (Fig. 4.21C-D). A similar outcome has been observed in *L.*

esculentum Mill. under salt stress and *C. annuum* under drought stress (Khan and AlZuaibr, 2022; Kaya and Shabala, 2023).

PAG treatment further suppressed glutamine synthetase and glutamate synthase activity by 19-32 and 29-38% in S1PAG-S3PAG treated okra seedlings compared to S1 and S3 seedlings (Fig. 4.21C-D). On the other hand, NaHS application to PAG-supplemented salt-stressed seedlings recovered the PAG-induced reduction in the activity of glutamine synthetase and glutamate synthase of salt-treated okra seedlings (Fig. 4.21C-D). The glutamine synthetase and glutamate synthase activity were recovered by 4-12 and 9-17%, respectively, in S1NPAG-S3NPAG seedlings compared to S1PAG and S3PAG okra seedlings (Fig. 4.21C-D). These findings demonstrate the protective effect of H_2S in regulating nitrogen assimilating enzymes.

Under stressful circumstances, when glutamine synthetase and glutamate synthase enzymes are not functioning appropriately, an alternate enzyme, glutamate dehydrogenase, converts ammonia to glutamate (Yang et al., 2013). Similarly, salt-stressed S1 and S3 okra seedlings demonstrate enhanced glutamate dehydrogenase activity by 23 and 57%, respectively, compared to the control (Fig. 4.21E). Similar results have been documented in *O. sativa* L. under nickel stress (Rizwan et al., 2019; Rizwan et al., 2022). In response to salt stress, glutamate dehydrogenase serves a dual protective role by promoting both glutamate production and ammonia detoxification, which helps in generating defensive metabolites (Ashraf et al., 2018).

On the other hand, the NaHS exposure reduced the glutamate dehydrogenase activity in S1N and S3N treated seedlings by 15 and 10%, respectively, then S1 and S3 seedlings (Fig. 4.21E) due to the restoration of the GS-GOGAT pathway (Raju and Prasad 2023). The application of HT revoked the impact of NaHS on glutamate dehydrogenase activity in okra seedlings. The glutamate dehydrogenase activity of okra seedlings was enhanced by 30 and 19% in S1NHT and S3NHT compared to S1N and S3N seedlings (Fig. 4.21E). Furthermore, PAG exposure also enhanced the glutamate dehydrogenase activity in S1PAG and S3PAG by 21 and 14%, than S1 and S3 salt-stressed okra seedlings (Fig. 4.21E). Further exposure of NaHS to S1PAG and S3PAG seedlings reduced the glutamate dehydrogenase activity by 3 and 4% in S1NPAG and S3NPAG seedlings, respectively (Fig. 4.21E). These findings highlight the regulatory role of H_2S in nitrogen metabolism under salt stress conditions, particularly in ammonia

ion assimilation and the modulation of key enzymes like glutamine synthetase, glutamate synthase, and glutamate dehydrogenase.

These enzymatic responses, along with observed morphological and biochemical changes, demonstrated the potential of NaHS in regulating salt stress responses in okra. As NaHS functions as H_2S donor, its ability to modulate endogenous H_2S levels was also evaluated in the study, consistent with previous research findings (Tang et al., 2020; Dawood et al., 2022a; Gautam et al., 2022).

4.20. Analysis of H_2S content in okra subjected to varied treatments

H_2S levels were raised under NaCl stress, suggesting its role as a secondary messenger to detect stress and trigger the physiological responses, through gene modulation (Lai et al., 2014). By considering the known role of H_2S in regulating plant responses to salt stress in this study, we investigated H_2S accumulation patterns in okra seedlings under various treatments (Fig. 4.22). The study examined the effects of NaCl, NaHS, HT, and PAG on H_2S content. Salt stress induced a significant increase in H_2S accumulation by 25 and 50% in S1 and S3 treated okra seedlings, respectively, compared to control seedlings (Fig. 4.22). The elevation in H_2S concentration under stress conditions indicated its involvement in stress perception and response mechanisms, as evidenced by subsequent modifications in morphological, physiological, and biochemical parameters of stress-exposed seedlings (Yang et al., 2023; Zhao et al., 2023).

NaHS treatment further enhanced H_2S levels, resulting in an additional increase of approximately 11% in S1N and S3N compared to salt-stressed seedlings (Fig. 4.22). Enhanced H_2S production on NaHS supplementation has earlier been documented in many crops (Min et al., 2016; Ding et al., 2019; Du et al., 2022; Yang et al., 2023; Zhang et al., 2023b). Therefore, as demonstrated in the current investigation, elevated H_2S levels in NaHS-treated okra were responsible for the enhanced morphological, biochemical, and antioxidant parameters that contributed to the alleviation of salt stress responses, thus facilitating salt stress tolerance.

However, applying H_2S scavenger HT substantially reduced H_2S content, causing 61 and 54% decrease in S1NHT and S3NHT seedlings relative to their NaHS-treated counterparts (Fig. 4.22). Studies have reported the reversal of NaHS-induced effects on plant growth parameters under stresses with HT exposure in several crops, including *S.*

indicum, *M. hupehensis*, and *C. pepo* (Amooaghaie and Enteshari 2017; Wei et al., 2019; Valivand and Amooaghaie, 2021a).

Similarly, PAG treatment led to a significant reduction in H_2S content, with decreases of 65 and 57% in S1PAG and S3PAG seedlings compared to their corresponding salt-stressed okra seedlings (Fig. 4.22). Notably, subsequent NaHS application counteracted the inhibitory effect of PAG in salt-stressed okra seedlings, resulting in an increase of H_2S content by 14 and 9% in S1NPAG and S3NPAG seedlings, respectively, relative to S1PAG and S3PAG seedlings (Fig. 4.22). The plant growth and stress responses were significantly altered on PAG exposure to NaCl-stressed *M. sativa*, *C. sativus* L. (Lai et al., 2014; Luo et al., 2023), cadmium and arsenate-stressed *M. sativa* and *O. sativa* L. seedlings (Mishra and Singh, 2021; Yang et al., 2021), and nickel-stressed *C. pepo* plants (Valivand et al., 2019a).

The experimental results showed that H_2S accumulation regulated by NaHS is crucial for maintaining robust and continuous signalling pathways that protect okra seedlings from NaCl toxicity. Measurement of H_2S levels confirms its function as a critical protective molecule in okra's stress response mechanism, potentially explaining the enhanced salt stress resistance observed in these seedlings.

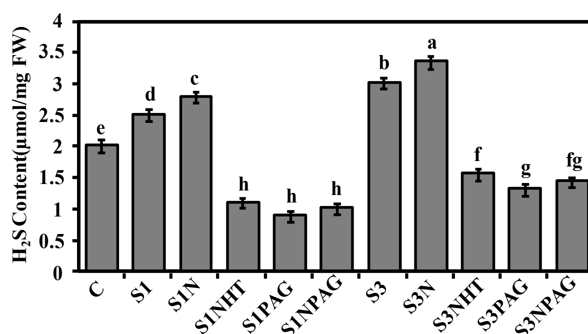

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Figure 4.22. NaHS is regarded as a potential H_2S donor. The bar graph illustrates the increased buildup of endogenous H_2S in salt-stressed okra following NaHS exposure. The hydroponic exogenous application of NaHS effectively elevated H_2S levels in okra, thereby enhancing salt stress tolerance.

So, in this study, we determined the effect of 100 µM NaHS concentration on the growth and development of okra under 25 and 75 mM of NaCl stress. Our results revealed that using exogenous NaHS could increase the endogenous H_2S content that

modulated the morphological and biochemical parameters of okra under NaCl stress, thereby promoting stress endurance. H_2S could prevent the content of pigment and carbohydrate from salt damage, thereby improving its growth attributes. Moreover, 100µM of exogenous NaHS could significantly improve the antioxidant potential, enzymatic antioxidant activity, including superoxide dismutase, catalase, and ascorbate peroxidase, and non-enzymatic antioxidants, including phenol and flavonoid, thereby reducing the oxidative stress, lipid peroxidation, hydrogen peroxide production, and ROS-induced damage to proteins. Meanwhile, H_2S could increase the efficiency of nitrogen metabolism in salt-stressed okra seedlings by inducing the nitrogen uptake and assimilation through improved nitrate reductase, nitrite reductase, glutamine synthetase, and glutamate synthase activity. Furthermore, HT and PAG demonstrated that H_2S is crucial for maintaining robust and continuous signaling pathways that help okra plants endure the toxic effects of NaCl stress.

Overall, Okra exhibited better salt tolerance at moderate NaCl concentration, but both the crops demonstrated severe damage at 75mM, signifying different salt sensitivities. The optimal H_2S donor concentration exhibited species specificity, with sponge gourd requiring 50µm NaHS, w.r.t. okra (100µM) for optimal effects. This significant difference suggests that okra has a lower endogenous production of H_2S or has a higher metabolic demand. This difference can also result from different cellular uptake/utilization mechanisms among these species. In addition to it, it has been observed that okra experienced more severe oxidative damage than sponge, indicating a greater membrane vulnerability to ROS in okra. This might be the reason that okra requires double the NaHS concentration for protection. This implies that H_2S supplementation strategies are crop-specific rather than universal.

Furthermore, okra demonstrated disproportionately severe responses to H_2S scavenging, suggesting greater dependence on exogenous H_2S supplementation for stress protection and less endogenous H_2S production/storage. Treatment with inhibitor (PAG) reveals that sponge gourd can utilise exogenous H_2S even when endogenous synthesis is blocked; however, okra's response to exogenous H_2S is highly dependent on intact endogenous H_2S production. This suggests fundamentally different H_2S utilisation/perception mechanisms between crops and thus emphasizes the need for crop-specific stress management protocols in saline environments.

Chapter 5

5. SUMMARY AND CONCLUSION

Various environmental stresses progressively jeopardize agricultural output in the face of rising global food security issues. Among them, soil salinity is one of the most important constraints worldwide. Because of its multifarious negative consequences on plant growth, development, and physiological processes, salinity stress represents a great limit on agricultural productivity. Plants suffer a cascade of negative reactions, including ionic imbalance, osmotic stress, and nutritional problems that together affect plant growth and agricultural yield. Salt-stressed plants exhibit oxidative damage resulting from reactive oxygen species overproduction, degradation of important photosynthetic pigments, severely compromised nutrient acquisition mechanisms, and impairment of nitrogen metabolism pathways. These stress-induced changes disrupt normal plant development and growth, showing up as stunted morphology, lower biomass accumulation, and finally lower crop output.

Recent developments in plant stress physiology studies have illuminated the remarkable potential of H_2S, given in the form of sodium NaHS, as a protective signaling molecule and stress-mitigating agent against salt-induced damage in a range of crop species. Building on this growing knowledge, the present work systematically investigated the protective mechanisms by which H_2S reduced NaCl-induced stress responses in sponge gourd and okra seedlings. Several physiological factors are covered in this comprehensive investigation, including morphological growth attributes, photosynthetic efficiency indicators, antioxidant defense systems, and nitrogen metabolism pathway.

To establish unequivocal evidence for the specific involvement of H_2S in salt stress alleviation in sponge gourd and okra, the experimental design included PAG, a selective inhibitor of endogenous H_2S biosynthesis, and HT, an effective H_2S scavenging agent. The inclusion of these compounds provided critical validation of the indispensable role of H_2S in orchestrating stress tolerance mechanisms in sponge gourd and okra under saline conditions.

Key highlights of study

- **Regulation of sponge gourd and okra growth under salt stress by NaHS**

Salt stress compromised plant growth by reducing the shoot and root length of sponge gourd and okra seedlings. Under saline conditions, the exogenous treatment of NaHS

showed significant efficiency in restoring the damaged growth characteristics. The H_2S-donor restored shoot length and root length by 14–22% in sponge gourd. Whereas in okra, shoot length was restored by 11–24% and root length by 20–47%. This restoration of the root enabled better nutrient uptake, therefore supporting general growth recovery, as evident by improved shoot length in the presence of salt stress.

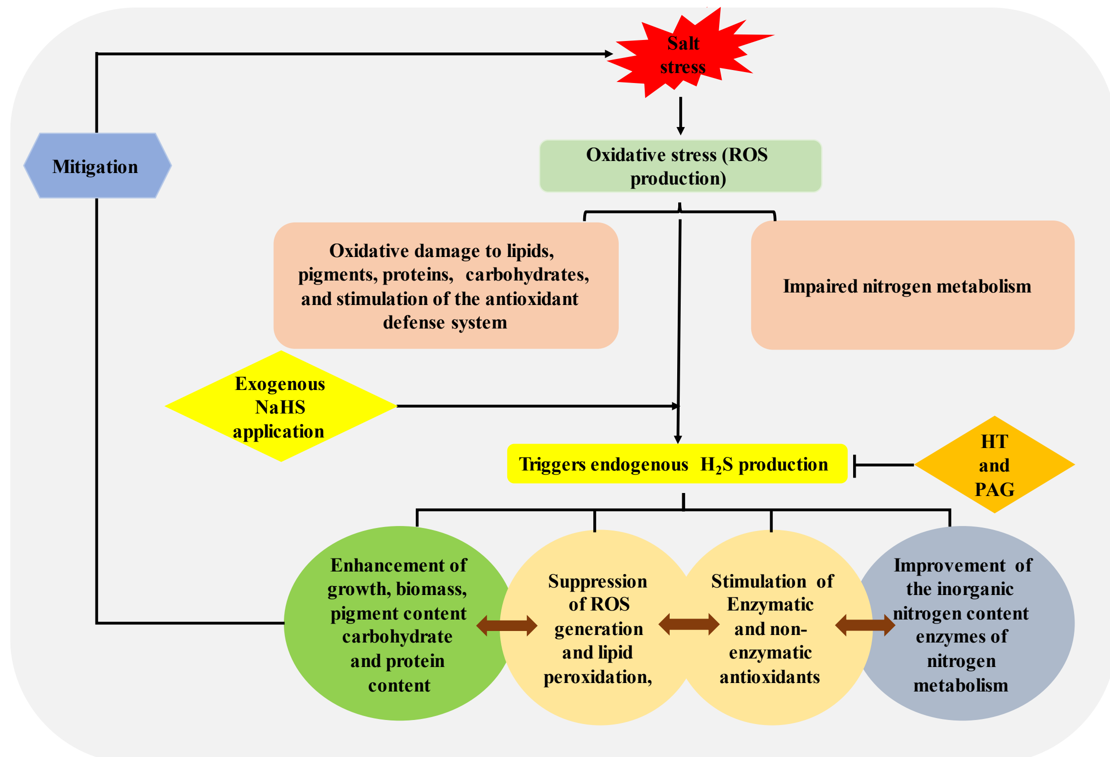

Figure 5. Represents the regulation of different parameters by H_2S under salt stress.

- **Regulation of biomass accumulation and photosynthetic pigments under salt stress by NaHS**

Under salt stress, photosynthetic efficiency is a major factor influencing plant output, with pigment integrity as a key indicator of photosynthetic apparatus functionality. NaCl stress caused a notable reduction in important photosynthetic pigments, including chlorophyll and carotenoids. Chlorophyll a & b were reduced by 13–42% in sponge gourd and 9–36% in okra. This pigment degradation seriously reduced the photosynthetic efficiency and light-harvesting ability of stressed plants. Reduced carbohydrate synthesis, decreased protein accumulation, and considerably lower fresh biomass were the downstream effects of hindered photosynthesis.

NaHS supplementation effectively maintained the integrity of photosynthetic pigments by inhibiting reactive oxygen species-induced degradation that is usually triggered under salt stress conditions. This protective effect on photosynthetic pigments improved photosynthetic efficiency, thereby improving the biosynthesis of carbohydrates, biomass, and proteins. The fresh weight and carbohydrate content of sponge gourd were restored by 20–24% and 20–29% compared to salt-stressed seedlings. Likewise, 100µM NaHS supplementation to salt-stressed okra seedlings restored fresh weight and carbohydrate content by 18–44% and 24-36%, respectively.

- **Mechanism of oxidative stress mitigation by H_2S under salt stress**

Salt stress increased the synthesis of reactive oxygen species, causing significant oxidative damage in plant tissues. Sponge gourd and okra under NaCl stress showed notably high levels of hydrogen peroxide and malondialdehyde. Maximum 241% increase in the hydrogen peroxide content was found in okra seedlings exposed to 75mM (S3) of salt stress compared to the control. These elevated oxidative stress indicators signaled substantial cellular dysfunction and structural damage under saline conditions.

NaHS-based H_2S supplementation triggered comprehensive improvement in plant antioxidant defense systems. The antioxidant defense potential was increased by 9% and 17–21% in NaHS exposed salt stressed sponge gourd and okra seedlings, respectively. The increment in antioxidant defense potential can be attributed to the upregulation of important enzymatic antioxidants like superoxide dismutase, catalase, and ascorbate peroxidase, along with higher synthesis of non-enzymatic antioxidant molecules such as total phenols and flavonoids. This multifaceted antioxidant reinforcement by H_2S effectively reduced oxidative stress damage and maintained cellular membrane integrity in salt-stressed seedlings.

- **NaHS-based regulation of nitrogen metabolism under salt stress**

Important metabolic processes extremely susceptible to disturbance caused by salt are nitrogen uptake and assimilation. Salt stress significantly decreased nitrate and nitrite levels in sponge gourd and okra by concurrently obstructing nitrogen absorption processes and downregulating the function of essential nitrogen metabolism enzymes, namely nitrate reductase and nitrite reductase. The nitrate reductase activity was

downregulated by 14-38% in sponge gourd and by 21-49% in okra. On the other hand, the activity of nitrite reductase gets reduced by 9-31% and 18-45% in sponge gourd and okra, respectively. Simultaneously, sponge gourd and okra subjected to NaCl stress demonstrated an aberrant accumulation of poisonous ammonia by 6-22% and 14-48%, respectively due to impaired activity of the glutamine synthetase-glutamate synthase (GS-GOGAT) enzyme pathway, which typically aids the assimilation of ammonia into amino acids.

H_2S supplementation via NaHS treatment markedly raised nitrate absorption efficiency by 35-44% and 25-53% and improved subsequent assimilation processes in sponge gourd and okra, respectively. This enhancement was accomplished by H_2S-induced activation of nitrogen metabolism enzymes, which successfully reinstated nitrogen homeostasis and facilitated protein biosynthesis under saline circumstances in sponge gourd and okra seedlings. The control of nitrogen metabolism is a crucial process by which H_2S enhances salt stress resistance.

- **Enzymatic pathway in detoxification and ammonia assimilation**

The research uncovered advanced enzymatic modifications related to nitrogen metabolism under stressful environments in sponge gourd and okra. Sponge gourd and okra exposed to salt stress demonstrated a substantial elevation in glutamate dehydrogenase enzyme activity by 15-39% and 23-57%, indicating a compensatory response to the compromised efficiency of the major GS-GOGAT ammonia assimilation pathway. This enzymatic adaptability enabled an alternate metabolic pathway for ammonia detoxification when primary channels are hindered by stress.

NaHS treatment significantly reinstated optimal functionality of the GS-GOGAT enzymatic system, thereby diminishing the plant's dependency on the alternative GDH route. The GDH activity thus decreases on NaHS exposure to salt-stressed seedlings by 11-17% and 10-15% in sponge gourd and okra, respectively. The rebalancing of the ammonia assimilation mechanism optimized nitrogen use efficiency under stressful circumstances, thereby facilitating ongoing growth and development despite saline environments.

- **Signaling role of H_2S under salt stress conditions in sponge gourd and okra**

The research presented compelling evidence for the role of H_2S as a molecular signal that orchestrated stress adaptation responses in sponge gourd and okra. Exogenous

administration of the H_2S donor, NaHS caused a substantial increase in endogenous H_2S concentration in the sponge gourd and okra seedlings by 11-12%. This finding reinforced the conceptualization of H_2S as a stress signal molecule in plant adaptive responses to environmental stresses.

The experimental treatments utilizing PAG (which inhibits endogenous H_2S synthesis) and HT (which scavenges accessible H_2S) completely negated the protective advantages conferred by NaHS administration in salt-stressed seedlings. This finding unequivocally validated the essential role and specificity of H_2S in modulating plant physiological responses under circumstances of salt stress.

This comprehensive investigation highlighted the crucial and multifaceted role of H_2S in mitigating the effects of salt stress on various physiological aspects in sponge gourd and okra seedlings. H_2S facilitated stress protection via several methods, including the improvement of morphological growth parameters, maintenance of photosynthetic machinery performance, strengthening of antioxidant defense systems, and intricate modulation of the nitrogen metabolism pathway.

Using H_2S turned out to be a very successful intervention method for raising endogenous H_2S levels in plant tissues, therefore reducing the negative consequences related to NaCl-induced stress. The observed reversal of protective effects upon experimental inhibition of H_2S production or activity by PAG or HT treatments supported the regulatory significance of H_2S even further.

In conclusion, the combined results showed that H_2S is a main signaling molecule in plant stress physiology with significant potential applications in agricultural practices. H_2S-based interventions represented a promising biotechnological approach for enhancing crop resilience and productivity under increasing soil salinity challenges, offering valuable strategies for sustainable agriculture in salt-affected regions worldwide.

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DETAILS OF PUBLICATION

1. ***Effect of sodium chloride mediated salt stress on seedling vigour and growth of okra (Abelmoschus esculentus L.) grown in hydroponics.***

Firdoos, A., Guleria, P., & Kumar, V. (2025). Effect of sodium chloride mediated salt stress on seedling vigour and growth of okra (Abelmoschus esculentus L.) grown in hydroponics. *Research on crops*, *26*(1) 10.31830/2348-7542.2025.ROC-1165

2. ***Biochar-based slow-release fertilizers for sustainable agriculture: a mechanistic overview on process development.***

Firdoos, A., Parbhakar, M., Guleria, P., & Kumar, V. (2025). Biochar-based slow-release fertilizers for sustainable agriculture: a mechanistic overview on process development. *Biochar Ecotechnology for Sustainable Agriculture and Environment*, 587-618.

3. ***Invitro NaHS exposure induced salt stress tolerance in sponge gourd.***

Firdoos, A., Guleria, P., & Kumar, V. (2025). In vitro NaHS Exposure Induced Salt Stress Tolerance in Sponge Gourd. *Russian Journal of Plant Physiology*, *72*(3), 75.

CONFERENCES

1. Orally presented in "Current Trends in Toxicology & 43rd Annual Meeting of Society of Toxicology (India) STOX-2024" (**ICCTT-2024**), held on 16th-19th October, 2024 in LPU, Punjab.

LOVELY PROFESSIONAL UNIVERSITY

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Certificate No. 355275

Certificate of Participation

This is to certify that Prof./Dr./Mr./Ms. **Assima Firdoos** of **School of Bioengineering and Biosciences, Lovely Professional University, Phagwara** participated and presented Poster/ Oral Presentation on the topic **Insights into the Biomolecular Regulatory Network of Hydrogen Sulfide in Sponge Gourd during Sodium Stress Amelioration** in **International Conference on Current Trends in Toxicology & 43rd Annual Meeting of Society of Toxicology, India, 2024 (STOX 2024): Human Health and Environmental Safety,** held on 16-18th October 2024 organized by School of Bioengineering and Biosciences in collaboration with Society of Toxicology (STOX), India, at Lovely Professional University, Punjab.

Date of Issue: 13-11-2024
Place : Phagwara (Punjab), India

Prepared by (Administrative Officer-Records)

Professor Neeta Raj Sharma, Convener, ICCTT 2024

Professor Alok Dhawan, President, STOX

Head of Faculty, Lovely Professional University

2. Orally presented in "4th International conference on global efforts on Agriculture, Forestry, Environment and Food security" (**GAFEF-2022**), held on 17th-19th September, 2022 in Nepal.

4th International Conference

Global Efforts on Agriculture, Forestry, Environment and Food Security

(Theme: Climate Change and Its Impact)

(GAFEF-2022)

CERTIFICATE

This is to certify that Prof./Dr./Mr./Ms. Assima Furdoos
of Lovely Professional University, Punjab, India
Actively participated/presented a paper (Oral/Poster) entitled Signal Transduction and Sodium Chloride Stress Amelioration by Hydrogen Sulphide in Solanum Melogena
As Delegate/Scholar in the "4th International Conference on "Global Efforts on Agriculture, Forestry, Environment and Food Security (Theme: Climate Change and Its Impact) (GAFEF-2022)" at Institute of Forestry, Tribhuvan University, Pokhara Campus, Pokhara, Nepal on September 17-19, 2022.

Prof. C. P. Singh, Conference Chairperson, President, AETDS, Former Professor, GBPUAT, Pantnagar, India

Dr. Arjun Prasad Bastola, Conference Director, Institute of Forestry, TU, Pokhra, Nepal

Dr. Santosh Marahatta, Organizing Secretary, AFU, Chitwan, Nepal

Dr. Wajid Hasan, Chief-Organizing Secretary, Secretary, AETDS, KVK Jehanabad, BAU Sabour, Bihar, India

AETDS/PC/GAFEF/2022/410

3. Poster presented in "**ICS-LOE 2021**", held on 17th-18th December 2021 in LPU, Punjab.

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INSTITUTE OF FOREST PRODUCTIVITY

Certificate No. 238868

Certificate of Participation

*This is to certify that **Prof./Dr./Mr./Ms. Assima Firdoos** of **Lovely Professional University** has participated in **Poster Presentation** on the topic entitled **Alternative Energy Solutions: From Waste to Value** in International Conference on **Sustainability: Life on Earth 2021 (ICS-LOE 2021)** held on 17-18 December 2021 organized by Department of Botany and Zoology, School of Bio-engineering and Biosciences, and Institute of Forest Productivity, Ranchi, Jharkhand at Lovely Professional University, Punjab.*

Date of Issue: 18-02-2022
Place of Issue: Phagwara (India)

Prepared by (Administrative Officer-Records)

Dr. Joydeep Dutta, Organizing Secretary, ICS-LOE, 2021

Dr. Neeta Raj Sharma, Convener, ICS-LOE, 2021

4. Participated in an International symposium on sustainability (**ISSUE-2022**), held from 13th-14th October, 2022 in Dehradun.

UPES UNIVERSITY OF TOMORROW

International Symposium on Sustainable Urban Environment (ISSUE-2022)

11-14 October, 2022

CERTIFICATE OF APPRECIATION

This certificate is presented to **Assima Firdoos and Dr. Parul Parihar** for **Sustainable Management of Agriculture Using Climate-resilient Strategies.** his/her participation at the International Symposium on Sustainable Urban Environment (ISSUE - 2022), organized by the Sustainability Cluster, UPES, Dehradun, India

Vice Chancellor, UPES & Chairperson

Dean (School of Engineering), UPES & Co-Patron

Cluster Head (Sustainability Cluster), UPES & Convenor

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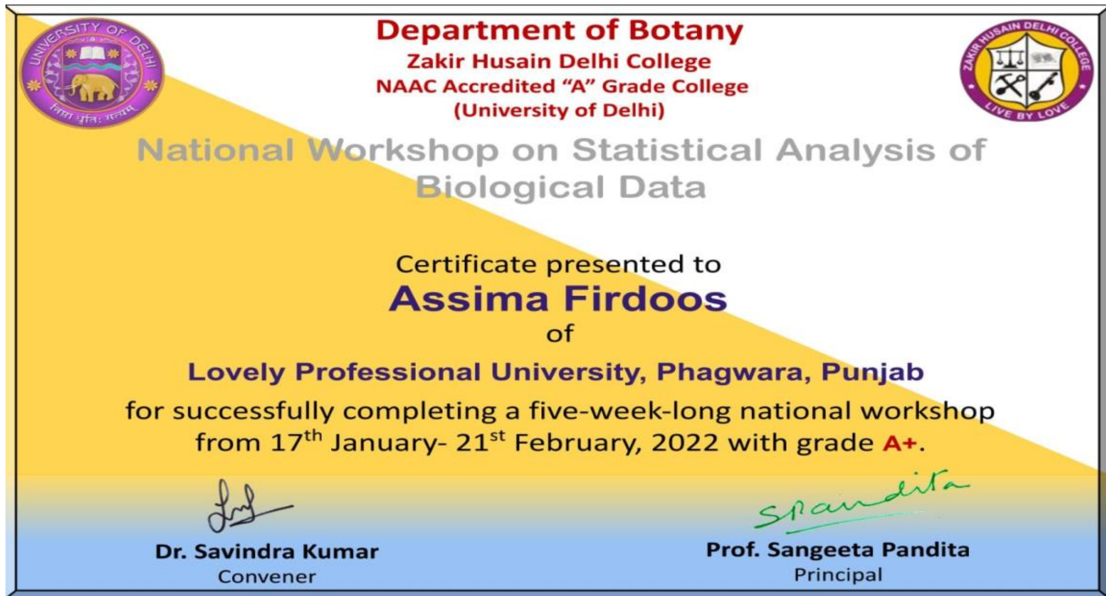
WORKSHOPS

1. Participated in the "National workshop on statistical analysis of biological data" from 17th January-21th February, 2022.



2. Participated in the "International workshop-cum-webinar on CRISPR Construct Design for efficient genome editing in plants" from 4th -6th December, 2024.

ANNEXURE

1. Certificates of plant identification.

भारतीय समवेत औषध संस्थान
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
केनाल रोड़, जम्मू – 180001
Indian Institute of Integrative Medicine
(Council of Scientific & Industrial Research)
Canal Road, Jammu Tawi - 180 001 (INDIA)

IIM/RRLH/2024/ Dated: 26-12-2024

Certificate for plant voucher submission and identification

Dry plant specimen was received from **Assima Firdoos Ph. D**. Scholar in Lovely Professional University, Jalandhar Punjab on 20th of November 2024. The provided samples were found to be twig of the ***Luffa aegyptiaca*** Mill family cucurbitaceae; Plant samples were collected and identified by the Assima Firdoos. After the taxonomic evaluation and comparison with the already submitted samples the identification found satisfactory. Received plant sample was prepared and has been submitted to Janaki Ammal Herbarium of CSIR-IIIM Jammu with the Accession number of **RRLH**: **30033.**

Dr. Manu Khajuria

Dr. Manu Khajuria
Scientist
Plant Sciences & Agrotechnology Division
CSIR-IIIM, Jammu

Phones : EPABX (0191) 2569000-06, (Director) 2569111, 2569222, 2569333 (Fax) : PME 2569019 (P/F) (CoA) 2569016-17 (F), PUR 2569025 (P/F), ACCTS 2569026, Website : (www.rrljammu.org)

भारतीय समवेत औषध संस्थान
(वैज्ञानिक तथा औद्योगिक अनुसंधान परिषद)
केनाल रोड़, जम्मू – 180001
Indian Institute of Integrative Medicine
(Council of Scientific & Industrial Research)
Canal Road, Jammu Tawi - 180 001 (INDIA)

IIM/RRLH/2024/ Dated: 20-05-2024

Certificate for plant voucher submission and identification

Dry plant specimen was received from **Assima Firdoos Ph. D**. Scholar in Lovely Professional University, Jalandhar Punjab on 20th of August 2024. The provided samples were found to be twig of the ***Abelmoschus esculentus*** (L.) Moench family Malvaceae. Plant samples were collected and identified by the Assima Firdoos. After the taxonomic evaluation and comparison with the already submitted samples the identification of all samples found satisfactory. Received plant sample was prepared and has been submitted to Janaki Ammal Herbarium of CSIR-IIIM Jammu with the Accession numbers of **RRLH**: **30032**

Dr. Manu Khajuria

Dr. Manu Khajuria
Scientist
Plant Sciences & Agrotechnology Division
CSIR-IIIM, Jammu

Phones : EPABX (0191) 2569000-06, (Director) 2569111, 2569222, 2569333 (Fax) : PME 2569019 (P/F) (CoA) 2569016-17 (F), PUR 2569025 (P/F), ACCTS 2569026, Website : (www.rrljammu.org)

2. List of Instruments used.

Instrument	Model No.
Spectrophotometer	Shimadzu corp. (80485)
Autoclave	NSW-227/0809310
Centrifuge	Neuation (iFuge MO8VT)
Centrifuge	Plasto Crafts (Rota 4R-V/F_M)
Centrifuge	Remi PR-24
Water bath	Labfit

Ice-flaking machine	Allied Froast
Incubator	Yorco (YSI-438)
Vortex shaker	Tarsons (CAT.3020)
pH meter	Labtronics (LT-10)
Laminar air flow	Rescholar
Electronic balance	Wensar-PGB220
Air pump	RS-A88
Distillation unit	Perfit
Hot air sterilizer	Yorco (YSI-431)
Deep freezer	Blue star (CHF500A)
Deep freezer	New Brunswick™ (U410-86)
Tissue culture rack	Local
Seed germinator	Labfit
Microwave	IFB (30SC3)
Temperature controller	Vista Biocell Pvt. Ltd.
Refrigerator	Samsung Rt-34