HYDROGEN SULFIDE AND NITRIC OXIDE SIGNAL INTEGRATION UNDER NaCl STRESS IN SOME MEMBERS OF THE FAMILY CUCURBITACEAE

Thesis Submitted for the Award of the Degree of

DOCTOR OF PHILOSOPHY

in

Botany

by

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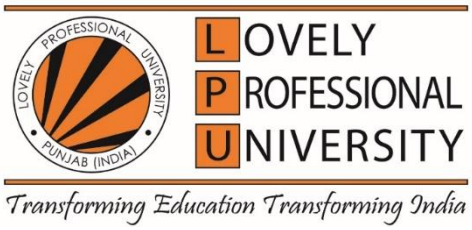
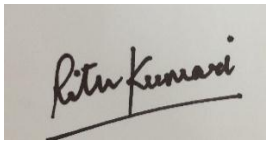


LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2025

DECLARATION

I, hereby declared that the presented work in the thesis entitled **"HYDROGEN SULFIDE AND NITRIC OXIDE SIGNAL INTEGRATION UNDER NaCl STRESS IN SOME MEMBERS OF THE FAMILY CUCURBITACEAE"** in fulfilment of degree of **Doctor of Philosophy (Ph. D.)** is outcome of research work carried out by me under the supervision of Dr. Gurmeen Rakhra, working as Assistant Professor, in the Department of Biochemistry, School of Bioengineering and Biosciences, Lovely Professional University, Punjab, India. In keeping with general practice of reporting scientific observations, due acknowledgements have been made whenever work described here has been based on findings of other investigator. This work has not been submitted in part or full to any other University or Institute for the award of any degree.



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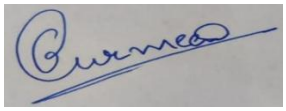
CERTIFICATE

This is to certify that the work reported in the Ph. D. thesis entitled **"HYDROGEN SULFIDE AND NITRIC OXIDE SIGNAL INTEGRATION UNDER NaCl STRESS IN SOME MEMBERS OF THE FAMILY CUCURBITACEAE**" submitted in fulfillment of the requirement for the award of degree of **Doctor of Philosophy (Ph.D.)** in Department of Botany, School of Bioengineering and Biosciences, is a research work carried out by Ritu Kumari (12008611), 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

Salt stress provides a considerable threat to plant growth and agricultural yield, affecting millions of hectares of land worldwide. The adverse effects of salt toxicity, primarily due to enhanced levels of NaCl in soil and water, disrupt essential physiological processes in plants, thereby negating the yields and soil quality. The present study thoroughly investigated the roles of H_2S and NO, facilitating salt stress tolerance in cucumber and bitter gourd. In this study, NaHS (sodium hydrogen sulfide), which is the donor of H_2S, and SNP (sodium nitroprusside), which is the donor of NO, were used as treatments for seedlings exposed to salt stress. Additionally, L-NAME and cPTIO, which are inhibitor and scavenger of NO respectively, were used to verify the involvement of NO in the presence of salinity.

NaHS and SNP supplementation, significantly enhanced morphological parameters including weight, height and pigments content, fostering robust growth under salt stress. These treatments also elevated endogenous H_2S and NO levels, activating nitrogen metabolism and antioxidative enzymes, which mitigated oxidative damage by reducing ROS and lipid peroxidation and this was also confirmed by *in-vivo* visualisation with NBT and DAB staining. However, applying the cPTIO and L-NAME disrupted the defense mechanisms, leading to excessive ROS accumulation, Na^+/K^+ imbalance, impaired nitrogen metabolism, and elevated oxidative stress markers. The decline in enzymatic antioxidants and protein content highlighted severe cellular damage thereby disrupting H_2S and NO signaling.

Overall, this research indicates that exogeneous application of H_2S and NO plays a crucial role in averting NaCl-induced toxicity by maintaining redox balance and enhancing immune defense mechanisms in plants. The cost-effective application of NaHS and SNP could serve as potent growth regulators, offering a sustainable solution to minimize crop losses in saline soils or under unavoidable stress conditions, thereby improving agricultural yields.

Keywords: Antioxidants, Hydrogen sulfide, Nitric Oxide, Physiology, Salinity, Stress

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A Ph.D. is not just an individual achievement, but it is the work of many minds, many hands, and many hearts

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Abbreviation:

µM - Micromole
$^1O_2$ - Singlet Oxygen
AAS - Atomic Absorption Spectrophotometer
ABA - Abscisic Acid
ANOVA - Analysis of Variance
AOA: Aminooxyacetic acid
APX - Ascorbate Peroxidase
AsA - Ascorbate
ATP - Adenosine Triphosphate
Ca - Calcium
Ca^{2+} - Calcium
$CaCl_2$ - Calcium chloride
CAT - Catalase
CDNB - 1-chloro, 2, 4-dinitro benzene
Cl^- - Chloride
cm - Centimetre
CO_2 - Carbon dioxide
cPTIO - 2-Phenyl-4,4,5,5-Tetramethylimidazoline-1-oxyl 3-oxide
Cu - Copper
$CuSO_4$ - Copper sulphate
DAB – Diaminobenzidine
DETA- 2,2-(Hydroxynitrosohydrazono)bis-ethanimine
DDW - Double Distilled Water
DHAR - Dehydroascorbate Reductase
DMRT - Duncan's Multiple Range Test
DNA - Deoxyribonucleic Acid
DNPGS - 2,4-dinitrophenyl-glutathione-S
DW - Dry Weight
E.C. - Electrical Conductivity
EDTA - Ethylenediaminetetraacetic Acid
ETC - Electron Transport Chain
ETHE1- ethylmalonic encephalopathy 1 protein
Fe - Iron
FW - Fresh Weight
GOGAT - Glutamate Synthase
GPX - Glutathione Peroxidase
GR - Glutathione Reductase
GS - Glutamine Synthetase
GST - Glutathione S-Transferase
GSNO- S-nitrosoglutathione
H_2O - Water
H_2SO_4 - Sulphuric acid
H_3BO_3 - Boric acid
H_2O_2 - Hydrogen Peroxide
H_2S - Hydrogen Sulfide

HCl - Hydrochloric acid
$HClO_4$ - Perchloric acid
HNO_3 - Nitric acid
K_2CO_3 - Potassium carbonate
K_2SO_4 - Potassium sulphate
K^+ - Potassium
KNO_3 - Potassium nitrate
KOH - Potassium hydroxide
L - Litre
LCD- L-cysteine desulfhydrase
L-NAME - Nω-Nitro-L-Arginine Methyl Ester
L-NMMA- N G -Monomethyl L-Arginine Monoacetate
M - Molar
MDA - Malondialdehyde
MDHAR - Monodehydroascorbate Reductase
mg - Milligram
Mg^{2+} - Magnesium
$MgCl_2$ - Magnesium chloride
$MgSO_4 \cdot H_2O$ - Hydrated magnesium sulphate
Min - Minute
ml - Millilitre
mm - Millimetre
mM - Millimole
Mn - Manganese
Na_2CO_3 - Sodium carbonate
Na^+ - Sodium
NaCl - Sodium Chloride
NaClO - Sodium hypochlorite
NAD^+ - Nicotinamide adenine dinucleotide radical
NADH - Reduced nicotinamide adenine dinucleotide
NADPH - Reduced nicotinamide adenine dinucleotide phosphate
NaHS - Sodium Hydrogen Sulfide
NaOH - Sodium hydroxide
NBT - Nitro Blue Tetrazolium
NEDD - N-(1-Naphthyl) ethylenediamine dihydrochloride
NH_4^+ - Ammonium
NiR - Nitrite Reductase
nm - Nanometre
nM - Nanomole
NO - Nitric Oxide
NO•- Nitric Oxide Radicle
NO_2^- - Nitrite
NO_3^- - Nitrate
NOS- Nitric oxide Synthase
NONOates- N-alkyl-N-nitrosohydroxylamines
NR - Nitrate Reductase
$O_2•^-$ - Superoxide Radical
O_2 - Oxygen
OEC - Oxygen-Evolving Complex
OH• - Hydroxyl Radical

$ONOO^-$ - Peroxynitrite
P – Phosphorus
PAG- DL-propargylglycine
PH - Plant Height
PSII - Photosystem II
PTIO- 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
RBOHD- Respiratory burst oxidase protein D
RNA - Ribonucleic Acid
RNS - Reactive Nitrogen Species
ROS - Reactive Oxygen Species
RuBisCo - Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase
S - Sulphur
SNP - Sodium Nitroprusside
SNAP: S-nitroso-N-acetylpenicillamine
SNF1- Sucrose non-ferme-1
SOD - Superoxide Dismutase
SOR - Superoxide Radical
SPSS - Statistical Package for the Social Sciences
TBA - Thiobarbituric acid
TCA - Trichloroacetic acid
U - Unit
UV-Vis - Ultraviolet-Visible (Spectrophotometer)
V - Volume
v/v - Volume by volume
W - Weight
w/v - Weight by volume
Zn - Zinc

CHAPTER 1

INTRODUCTION

“Sustainability is no longer about doing less harm. It’s about doing better.”
~ Jochen Zeitz

Salinity, as an abiotic stress in plants, refers to high levels of soluble salts, primarily NaCl in the soil or irrigation water. Saline soils have high levels of soluble salts (NaCl, Na_2SO_4, Cl^-, $CaSO_4$, and $MgSO_4^+$), which impede plant growth by reducing available water and interfering with nutrient uptake (Bui *et al.,* 2017). Electrical conductivity is normally high in saline soils, leading to poor health conditions in plants and less agricultural output. The sodic soils have high sodium ion content that contributes to poor soil structure, low infiltration, and deficient nutrition. Paramount salt concentration in the soil leads to a drastic change in the overall health of a plant by disrupting its osmotic balance, water absorption, nutrient uptake and ion transport (Li *et al.,* 2020). Notably, salinity has devasted about two-thirds of the world's agricultural land, affecting approx. 1,120 million hectares globally. However, it's noteworthy that a significant portion (~85%) of the affected land experiences only mild salt concentrations whereas the rest 15% faces severe salt concentrations limiting crop cultivation drastically (Wicke *et al.,* 2011). In India, nearly 7 million hectares are affected by saline soil, primarily in the Indo-Gangetic Basin and collectively accounting for around 75% of saline and sodic soil (Singh, 2009; Singh *et al.,* 2009, 2015, 2020a). Recent assessments indicate that approximately 6.7 million hectares of land in India are currently affected by salinity, with projections suggesting an increase to 11 million hectares by 2030 due to factors like climate change, poor irrigation practices, and inadequate drainage systems (Gogoi *et al.,* 2024). Moreover, mismanagement of natural resources, reduced water supply, and population growth can also lead to increased soil salinity in crop production, which in turn heightens the demand for food and fodder (Chen *et al.,* 2020; Varzakas & Smaoui, 2024).

The primary cause of salinity in agricultural land is the weathering of parental rocks, releasing various salts such as sodium chloride, calcium chloride, and magnesium chloride in excess amounts, along with small amounts of sulphates and carbonates (Golezani *et al.,* 2019; Singh, 2022b; Singh & Prasad, 2015). Others causes include discharge from industries like paint and dye, leaching from landfills, irregular irrigation, and unnecessary applications of fertilizer and pesticides (Singh & Prasad, 2015a; Martínez *et al.,* 2010; Santa-María *et al.,* 2015; Singh *et al.,* 2020a). NaCl detrimentally affects various processes in plants, including photosynthesis, antioxidant mechanisms, mineral nutrient regulation, accumulation of osmolytes, and hormonal signalling (Singh *et al.,* 2015). Stress triggers a rapid surge in the generation of various ROS like H_2O_2,$^{\cdot}$ OH, $O_2^{\cdot-}$, and RNS such as NO, $ONOO^{-}$, and $NO^{\cdot}$, either sequentially or concurrently like H_2S, H_2O_2, NO, and ethylene, to regulate ROS levels. Despite the presence

of different defence mechanisms, salinity remains a significant global threat to crop yields. Annual crops, which are often not salt-tolerant, are typically restricted to places with constant rainfall. Effective salinity defence requires rapid activation of the plant's defence system, which is dependent on accurate stress signal reception and processing. One of the best approaches employed by plants to negate the harmful effects of salinity is the external administration of signalling molecules like CO, H_2O_2, H_2S, NO, Ca^{2+} and phytohormones etc in plant metabolic processes (Jiang *et al.,* 2019; Paul & Roychoudhury, 2020). These molecules not only help the plant to grow and develop but also helps to adapt according to environmental conditions.

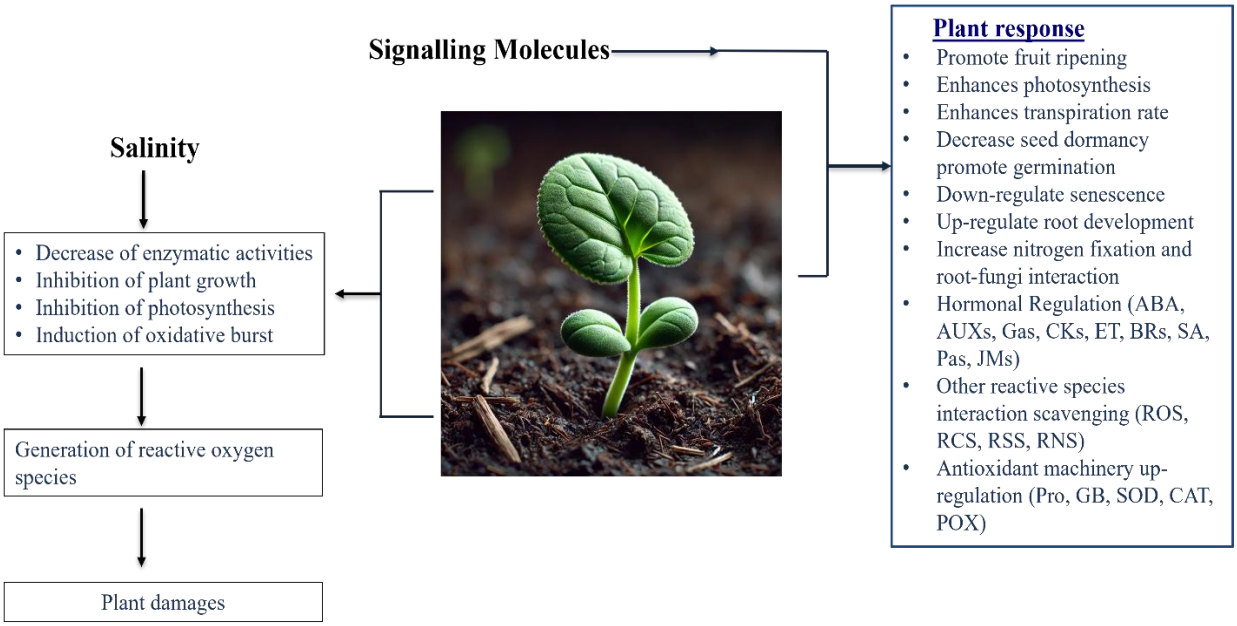


Figure 1.1: Diagrammatic illustration of the impacts of salinity and signalling molecules on plant growth and development

NO is an important messenger in signalling, as it aids signal transduction and helps plants cope with abiotic stressors. NO is a remarkably versatile molecule, existing as a diatomic gas with a neutral charge and exhibiting lipophilic properties, facilitating its role as an ideal signaling molecule capable of rapid diffusion across membranes (Castello *et al.,* 2019; Goretski & Hollocher, 1988). Initially, NO was regarded as merely a signaling molecule and reactive species. However, it is now recognized as a crucial factor in plant growth and responses to various environmental stresses, including nutrient deficiency, salinity, and heavy metal toxicity (Hasanuzzaman *et al.,* 2018; Alsolami, *et al.,* 2020; Sharma *et al.,* 2020). NO is widely acknowledged for its significant role in regulating the expression of ammonium (NH_4^+) transporters, thereby facilitating NH_4^+ uptake and highlighting its involvement in nitrogen absorption and metabolism (Huang *et al.,* 2020; Kumari *et al.,* 2024). It also acts as an anti-senescence signal, which delays the ageing of plant tissues and maintains chlorophyll retention

along with cellular function (Modolo *et al.,* 2017; Duan *et al.,* 2015; Corpas *et al.,* 2019; Sami *et al.,* 2018). NO regulates cytoskeleton organization, influence root hair growth and development for efficient water and nutrient absorption. Additionally, NO assists in alternation of metabolic process, wound healing, UV-B stress response, and coping with high-light irradiance in some plant species such as *Dasycladus vermicularis, Chlorella pyrenoidosa,* and *Ostreococcus tauri* (Abogadallah *et al.,* 2010; Zhang *et al.,* 2010; Foresi *et al.,* 2010; Jacobs *et al.,* 2006) In *Oryza sativa*, the senescence-induced protein loss induced by H_2O_2 treatment is reversed by exogenous NO.

The apoplast, chloroplast, cytoplasm, mitochondria, peroxisome, mitochondria and plasma membrane are the primary sources of the generation of NO in plants. Approximately eight oxidative and reductive pathways are intricate in the production of NO in plants, as identified by a number of studies: (a) Oxidative pathways: The oxidation of L-arginine, polyamines, and hydroxylamines generates NO with the help of a NOS(b) Reductive pathways: NO reductase, XOR, NR, cytochrome-c oxidase, or cytochrome-c reductase assist the reduction of nitrite by plasma membrane-bound nitrite, which produces NO (Fatima *et al.,* 2022b). Exogenously applied NO functions as a signaling molecule, enhances growth, germination, and photosynthesis, and prolongs the shelf life of fruits and vegetables.

After NO, hydrogen sulfide (H_2S) also emerged as an naturally occurring gaseous messenger (Yamasaki & Cohen, 2016). Some noteworthy research highlighted the fact that coupling NO with other signalling molecules like H_2S significantly lessened the harmful impacts of salinity and improved plants' overall development (Alnusairi *et al.,* 2021; Khan *et al.,* 2016; Khan *et al.,* 2020). Numerous studies indicate that H_2S is essential for many facets of plant development and growth, encompassing root architecture, seed germination, stomatal movement, photosynthesis, and the modulation of antioxidant levels (SOD, CAT, GST, APX, GPX) under both normal and stressful conditions, provided it is present within appropriate concentrations (Raju & Prasad, 2021a; Wang *et al.,* 2020). Besides, the K^+/Na^+ ratio in alfalfa roots improved due to exogenous H_2S, which led to the inhibition of K^+ leakage under saline conditions (Lai *et al.,* 2014). In another study, barley seedlings treated with the H_2S donor, NaHS, increased their ability to withstand salt by expressing more high-affinity K^+ transporter channel proteins (Chen *et al.,* 2015a). Inconclusively, H_2S provides tolerance to plants by enhancing antioxidant potential, maintaining ion homeostasis, reducing the uptake of Na^+ and Cl^- ions, and triggering signalling molecules (Ahmed *et al.,* 2021; da-Silva, 2017; AlSolami, *et al.,* 2020; Xu *et al.,* 2019; Zhao *et al.,* 2022).

Plants not only absorb H_2S from the atmosphere through leaves but also produce themselves. H_2S synthesis takes place frequently in chloroplast and partially in mitochondria and cytosol (Aroca *et al.* 2018). Additionally, a number of studies show that H_2S plays a key role in a some physiological processes, including fruit ripening, stomatal conductance, rhizobium-legume symbiosis, and post-harvest quality enhancement (Corpas *et al.,* 2023; Nabi *et al.,* 2019). It is formed in chloroplasts from sulfite by the enzyme sulfite reductase, whereas in the cytosol it is formed through the action of cystathionine synthase and cystathionine lyase. In mitochondria, its formation results from cyanide detoxification through the action of β-cyanoalanine synthase and O-acetylserine (thiol) lyase (*Yamasaki et al. 2019*). Despite the documented positive effects of H_2S on plant performance under abiotic stress, there is a paucity of research comparing its function across different plant systems. By activating antioxidant defence mechanisms to reduce oxidative stress, the application of exogenous H_2S has been demonstrated to provide protection against abiotic stressors like salinity, heavy metal toxicity, drought, and severe temperatures(Ni *et al.,* 2016; Raju & Prasad, 2023; Tian *et al.,* 2016).

The interaction between NO and H_2S in plant signaling under abiotic stress is a fascinating area of research in plant biology. NO and H_2S have been found to interact and influence each other's signaling pathways under abiotic stress conditions (Fatima *et al.,* 2022b). They can act synergistically or antagonistically depending on the specific stress and the context of the plant response. Additionally, beyond their signaling roles, the nucleophilic nature of H_2S and the electrophilic nature of NO suggest that the interaction between these gaseous molecules can form a new intermediate in enzymatic pathways such as those involving sulfide: quinone reductase. These intermediates have potential implications for enzyme regulation, signaling pathways, and redox balance in biological systems. Although NO was reported to enhance H_2S production in salt-treated plants, thus stimulating antioxidant defense, the molecular mechanisms of NO-H_2S crosstalk are not well defined (Gadelha 2017; Sehar *et al.,* 2019; Fatima *et al.,* 2022; Singh *et al.,* 2020; Kharbech *et al.,* 2022). NO operates upstream of hydrogen sulfide and takes a part in promoting antioxidant defense mechanisms in plants (Chen *et al.,* 2015a; Kharbech *et al.,* 2022; Singh *et al.,* 2020b). Besides, dose-response and threshold levels of NO for optimum H_2S synthesis, species-specific responses, long-term effects of NO and H_2S signaling, and influence of environmental factors on this interaction have been taken less into consideration.

Consequently, in light of the lacunae in this field, it is essential that additional studies be carried out at the laboratory and field stages to investigate the actual relationship between NO and H_2S. This might provide insight into the redox regulation of gasotransmitters, potentially uncovering

novel therapeutic targets and enhancing our understanding of their physiological roles. Consequently, the global community is anticipating the manner in which plant biologists will leverage the potential of signalling molecules in future agriculture to adequately sustain the 7.5 billion people.

Several plants in the Cucurbitaceae family, like cucumber and bitter gourd, are particularly sensitive to salt stress. This sensitivity means they can suffer damage and show reduced growth when exposed to high salt levels in the soil (Wu *et al.,* 2020). In recent years, there has been noticeable economic losses in the production of cucumber and bitter gourd due to salt stress, which lowers both their yield and nutritional quality (Singh *et al.,* 2022a; Siddiqui *et al.,* 2020). It could be promising to explore how H_2S and NO may help protect these plants from damage caused by salt (NaCl). Based upon the studies in mammals, we have investigated the role of different signalling molecules using the donors, inhibiters, or scavengers of NO. For this purpose, we used L-NAME, an inhibitor of NO, and cPTIO, a scavenger of NO, along with NaHS, and SNP. With this approach, we aim to better understand how NO and H_2S work together to help cucumber and bitter gourd plants withstand salt stress.

Therefore, to develop a comprehensive understanding of the integrated crosstalk between H_2S and NO and how NO contributes to improving the physiological and biochemical responses of cucumber and bitter gourd seedlings (Cucurbitaceae family) under NaCl stress, the following objectives are outlined:

1. Analysis of growth pattern and photosynthetic pigments in test seedlings.
2. Analysis of inorganic nitrogen content and co-enzymes involved in nitrate and ammonia assimilation in test seedlings.
3. Evaluation of oxidative damage and antioxidant activity in test seedlings.
4. Elucidation of crosstalk mechanism with nitric oxide.

CHAPTER 2

REVIEW OF LITERATURE

Broadening the Scope: Salinity's Comprehensive Outlook

In the current scenario, the agriculture sector faces immense pressure to increase food production by 60% to fulfil the needs of the growing inhabitants expected to surpass 9.3 billion by 2050 (Kumari *et al.,* 2024; Mir *et al.,* 2024). Concurrently, salinity levels are projected to rise to 20 M ha in India by the same year. This rise in salinity, coupled with the continual conversion of fertile land into non-agrion areas, diminishes the available arable land for cultivation. Consequently, there's a significant decline in the production of vital cash crops like vegetables, which are crucial components of our diet providing essential micronutrients such as zinc, iron, magnesium, vitamins (B and C), and fibres (Raju & Prasad, 2023).

High salt levels present a formidable obstacle to plant growth and development, severely impairing processes such as seed germination and biomass accumulation. Salinity primarily affects root functioning by disrupting water uptake and the absorption of essential nutrients, which are fundamental for sustaining healthy plant physiology (Kumari *et al.,* 2024, 2025). Among the salts, sodium chloride (NaCl) is particularly detrimental, interfering with numerous vital plant processes including photosynthesis, antioxidant defense systems, nutrient balance, osmolyte synthesis, and hormonal signaling (Singh *et al.,* 2015). One of the central challenges posed by salt stress is the excessive generation of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and superoxide anions ($O_2^{\bullet -}$), alongside reactive nitrogen species (RNS) like nitric oxide (NO), peroxynitrite ($ONOO^-$), and nitric oxide radicals (NO•). These reactive molecules cause oxidative damage to lipids, proteins, nucleic acids, and cellular membranes, ultimately compromising plant viability (Hasanuzzaman *et al.,* 2020).

To survive under such harsh conditions, plants have developed intricate adaptive mechanisms. A key strategy involves the selective accumulation of ions such as sodium (Na^+), chloride (Cl^-), and potassium (K^+), which are critical in maintaining cellular ion homeostasis and osmotic balance. Additionally, plants synthesize and accumulate compatible solutes including amino acids, betaines, proline, and soluble carbohydrates that function as osmoprotectants. These molecules help lower the osmotic potential within cells, thereby enabling water retention and protecting cellular structures from salt-induced dehydration (Khan *et al.,* 2023; Singh & Prasad, 2021). Crucially, the plant's ability to rapidly detect salinity stress and activate appropriate defense mechanisms largely determines its tolerance level. Early stress perception triggers complex signaling cascades that enhance antioxidant enzyme activities, regulate ion transport channels, stabilize proteins, and induce the expression of stress-responsive genes. This multifaceted response helps mitigate the oxidative stress caused by ROS and RNS, preserving

cellular integrity and metabolic function (Kumari *et al.,* 2024, 2025). Moreover, osmotic adjustments allow cells to maintain turgor pressure, ensuring continued growth and development despite external salinity challenges.

In addition to endogenous responses, the application of external signaling molecules has emerged as an effective approach to enhance salt tolerance in plants. Molecules such as carbon monoxide (CO), hydrogen peroxide (H_2O_2), hydrogen sulfide (H_2S), nitric oxide (NO), calcium ions (Ca^{2+}), and phytohormones play pivotal roles in orchestrating stress responses. These compounds act as secondary messengers, modulating gene expression, enzyme activity, and metabolic pathways crucial for plant adaptation. For example, nitric oxide and hydrogen sulfide have been shown to regulate antioxidant defenses, maintain ion homeostasis, and improve osmolyte accumulation under salt stress. Calcium ions function as universal signaling molecules that translate external stimuli into cellular responses, enhancing the plant's capacity to withstand salinity (Jiang *et al.,* 2019; Kaya *et al.,* 2023a; Paul & Roychoudhury, 2020).

Collectively, these signaling molecules fine-tune plant metabolic activities, balancing growth and stress mitigation. Their interplay facilitates dynamic adjustments, allowing plants not only to survive but also to maintain productivity under otherwise detrimental saline conditions. Understanding and harnessing these molecular signals holds great promise for developing salt-tolerant crops, which is increasingly important in the face of global soil salinization and climate change. Advanced research into these signaling pathways continues to reveal new insights, offering potential strategies for genetic improvement and agronomic management to safeguard food security in saline-affected regions.

Involvement of NO in plants under abiotic stress conditions:

Plants, being sessile organisms, encounter various environmental stressors that significantly impact their growth and productivity. These stress conditions lead to the generation of reactive chemical species including reactive oxygen species (ROS), reactive nitrogen species (RNS), reactive sulfur species (RSS), and reactive carbonyl species (RCS) (Zhou 2021). Among them, NO, classified under RNS, has emerged as a key signaling molecule due to its roles in modulating cellular metabolism, redox balance, and defense responses (Kumari 2024).

Over time, the involvement of NO had improved from being a signalling molecule and reactive species to being a necessary element for plant growth and responses to environmental cues including drought stress, salt stress, heavy metal stress, water deprivation conditions leading to drought and salinity stress (Hasanuzzaman *et al.,* 2018; Khan, AlSolami, *et al.,* 2020; Montilla-*et al.,* 2017; Sharma *et al.,* 2020b). Additionally, NO is also implicated in responses to plant-

rhizobium infection, fungal interactions, and many other symbiotic associations (He *et al.,* 2019; Jedelská *et al.,* 2021; Martínez-Medina *et al.,* 2019).

Nitric Oxide's Triple Play in Plants: Donors, Inhibitors, and Scavengers

NO is a crucial signaling molecule in plant physiology, implied in regulating growth, development, and responses to various abiotic and biotic stresses. However, due to the reactive and gaseous nature of NO, its direct application in plant systems is impractical. To overcome this, NO donors are widely utilized in research to mimic endogenous NO production and study NO-mediated responses under controlled conditions. These chemical compounds release NO in specific physiological or environmental contexts, making them valuable tools for dissecting the roles of NO in plant signaling networks (Tan *et al.,* 2013).

Commonly used NO donors in plant research include SNP, SNAP, GSNO, and a class of synthetic compounds known as NONOates. Each of these donors differ in their kinetics of NO release and the specific chemical form of NO delivered. For instance, SNP primarily releases nitrosonium ions (NO^+) and, under certain environmental conditions, can also produce NO radicals (NO•) (Arasimowicz *et al.,* 2011). In contrast, SNAP and GSNO are known to release NO• radicals, with GSNO providing a relatively longer NO-release half-life of around 7 hours, offering sustained NO availability in experimental setups. NONOates are designed to release NO steadily at controlled rates under physiological pH, while organic nitrates (NO_3^-) can act as indirect NO donors through metabolic reduction pathways in plant tissues (Arasimowicz *et al.,* 2011).

These variations in the chemical form and kinetics of NO release among different donors significantly influence their biological activity, application strategies, and interpretation of stress physiology experiments. For example, the form of NO released effects its interaction with target biomolecules such as proteins, reactive oxygen species (ROS), and other signaling intermediates, thereby modulating specific physiological processes like antioxidant defense activation, stomatal movement, and stress signal transduction. Thus, selecting an appropriate NO donor based on its release characteristics and experimental objectives is crucial for accurately investigating NO's roles in plant stress physiology and signaling.

The studies of NOS (nitric oxide synthase) inhibitors in plant systems also provide evidence for the existence of NOS-like activity in plants. NOS are homodimeric enzymes that convert L-arginine to NO. NOS inhibitors L-NAME and L-NMMA, are commonly applied as inhibitors of NOS and these mainly consist of arginine analogues that compete for the active site of the enzymes. The arginine analogues are capable of interfering with iron-dependent

systems, which might influence other pathways in addition to inhibiting NO synthase (Fatima *et al.,* 2022b; Habib & Ali, 2011). When the availability of L-arginine decreases, these enzymes (NOS) further produce superoxide anion and NO, which might contribute to the formation of peroxynitrite (Wendehenne *et al.,* 2001). NOS inhibitors have been applied in different environments on various plant models over the past two decades. Another significant inhibitor of the NR pathway is sodium tungstate. This compound serves as a non-specific NR inhibitor, effectively retarding NO production by competitively binding to the enzyme at its active site. It inactivates NR by impeding molybdate-dependent activity in plants (Fatima *et al.,* 2022a).
NO scavengers have been used in plant systems to confirm that NO is involved in pathways activated by stimuli (D'alessandro *et al.,* 2013). cPTIO, PTIO, are mostly used as scavengers of NO. cPTIO and PTIO scavenge NO to prevent it from interacting with target molecules thereby affecting various physiological processes and downstream signalling pathways considerably (Hogg *et al.,* 2017; Sueishi *et al.,* 2011). It is well known that NO works with other signaling pathways to control gene expression and strengthen the defense system under stressful circumstances.

Involvement of H_2S under abiotic stress conditions in plants

Similar to NO, H_2S has emerged as a critical gaseous signaling molecule involved in plant adaptation to various stresses. Initially considered a toxic by-product, H_2S is now recognized as part of the broader family of reactive sulfur species (RSS), with significant influence on plant growth, development, and environmental stress responses. Abiotic stresses such as drought, salinity, heavy metals, heat, chilling, and osmotic stress trigger the endogenous generation of H_2S in plants, which then functions as a regulator of physiological and molecular responses (Kaya & Ashraf, 2020; Kumari *et al.,* 2025). The regulatory role of H_2S includes activation of antioxidant enzymes, modulation of gene expression, preservation of ion homeostasis, and enhancement of osmoprotectant synthesis, thereby mitigating the detrimental effects of stress-induced ROS.

H_2S Signaling: The Three Pillars of Control – Donors, Inhibitors, Scavengers

Due to the gaseous and reactive nature of H_2S, direct application in experimental systems is impractical. To address this, various H_2S donors are used in plant research to simulate endogenous H_2S production. The most employed donor is Sodium Hydrosulfide (NaHS), which rapidly releases H_2S upon dissolution and is widely used to assess stress mitigation mechanisms in controlled environments (Guo *et al.,* 2018; Li *et al.,* 2014 & 2020). Other donors include GYY4137, a slow-releasing H_2S donor that provides sustained exposure and is more suitable for long-term studies. These donors have been shown to improve plant resilience by modulating redox status, enhancing chlorophyll retention, promoting antioxidant activity, and supporting hormonal balance, particularly under salt and drought stress conditions (Pandey & Gautam, 2020). The form, release rate, and concentration of H_2S delivered by these donors significantly impact their physiological effects, emphasizing the importance of selecting the appropriate donor for specific experimental objectives.

Although less studied than NO inhibitors, H_2S inhibitors help elucidate the specific roles of H_2S in plant stress physiology. Hypotaurine is commonly used as an H_2S inhibitor due to its ability to react with and neutralize H_2S molecules. The application of inhibitors, confirm that observed physiological effects are directly mediated by H_2S rather than secondary pathways. Moreover, PAG and AOA are inhibitors of enzymes involved in H_2S biosynthesis, such as LCD. By inhibiting these enzymes, researchers can evaluate how reduced endogenous H_2S affects plant responses to salinity, drought, and oxidative stress (Shen *et al.,* 2013; Yamasaki *et al.,* 2019). These studies demonstrate that inhibition of H_2S production typically results in reduced antioxidant capacity, impaired ion homeostasis, and greater oxidative damage, thereby reinforcing the protective role of H_2S in stress adaptation.

In parallel to NO scavengers like cPTIO, hemoglobin (Hb) is frequently used as H_2S scavenger in plant systems. This molecule binds free H_2S, thereby preventing it from participating in signaling or physiological pathways. By scavenging H_2S in treated plants, researchers can observe altered responses to abiotic stress, confirming H_2S's involvement in mitigating stress effects. Such scavenger-based experiments have shown that depletion of H_2S results in reduced chlorophyll content, impaired photosynthesis, increased electrolyte leakage, and weakened antioxidant defense mechanisms (Li *et al.,* 2020; Christou *et al.,* 2013). Thus, scavengers serve as vital tools for dissecting the molecular basis of H_2S signaling in plant stress responses.

Dual Dynamics of H_2S and NO in Plants under stress:

H_2S and NO are small signaling molecules that have emerged as crucial regulators of plant responses to environmental stresses. Their interaction often referred to as cross-talk plays a significant role in modulating key physiological and molecular processes that enhance plant resilience. In many cases, H_2S and NO function cooperatively, strengthening the plant's antioxidant defense system, maintaining redox balance, and supporting cellular homeostasis. However, their relationship is not always harmonious; under certain conditions, their interaction may be antagonistic, with one gas mitigating or altering the effects of the other. Importantly, both molecules influence protein activity through post-translational modifications persulfidation by H_2S and S-nitrosylation by NO frequently targeting the same cysteine residues. This biochemical competition shapes downstream signaling and stress responses, making the H_2S-NO interaction a complex yet vital aspect of plant stress physiology (Aroca *et al.,* 2015; Corpas & Palma, 2020; Hancock & Whiteman, 2016). Salinity stress poses a major challenge to plant growth by disrupting ionic balance and generating oxidative stress. The coordinated action of H_2S and NO has been found to alleviate these harmful effects. When applied together, these gasotransmitters enhance the activity of key antioxidant enzymes such as SOD, CAT, and APX, which collectively help reduce the accumulation of ROS and limit lipid peroxidation (Kaya *et al.,* 2019; Shi *et al.,* 2015). This biochemical support preserves membrane integrity and promotes cellular stability under salt stress. Additionally, H_2S and NO contribute to the regulation of ion transport mechanisms, particularly by restricting sodium (Na^+) uptake and improving potassium (K^+) retention. These processes help sustain osmotic balance and improve water use efficiency. At the molecular level, the H_2S-NO interaction influences the expression of stress-responsive genes, leading to better photosynthetic performance and enhanced overall tolerance to saline environments. Together, their cross-talk forms a dynamic and adaptive system that enables plants to cope more effectively with salinity-induced challenges.

Table 2.1: Common donors, inhibitors, and scavengers of H_2S and NO, their modes of action, physiological effects in plants

Category	Signaling Molecule	Common Compounds	Mode of Action	Effects in Plants	Key References
Donors	NO	Sodium Nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), S-nitrosoglutathione (GSNO), NONOates	Controlled NO release under physiological conditions	Enhances antioxidant defense, regulates ion homeostasis, modulates gene expression under salinity and drought stress	Arasimowicz 2011; Tan 2013
	H_2S	Sodium Hydrosulfide (NaHS), GYY4137	NaHS or sustained GYY4137 H_2S release	Activates antioxidant enzymes, maintains ion balance, delays senescence, and improves water status	Guo *et al.,* 2018; Li *et al.,* 2020
Inhibitors	NO	L-NAME, L-NMMA, Sodium Tungstate	Inhibit NOS-like activity (L-NAME, L-NMMA) and nitrate reductase pathway (Sodium Tungstate)	Reduces NO production; used to confirm NO-dependent processes under stress	Arasimowicz 2011; Zhou 2021
	H_2S	DL-propargylglycine (PAG), Aminooxyacetic acid (AOA)	Inhibit L-cysteine desulfhydrase (LCD) and other H_2S-producing enzymes	Reduces H_2S-mediated stress tolerance responses	Shen *et al.,* 2013; Yamasaki *et al.,* 2019
Scavengers	NO	cPTIO, PTIO	React with and neutralize NO radicals	Blocks NO signaling; used to validate NO involvement in stress responses	Arasimowicz 2011; Hasanuzzaman *et al.,* 2020
	H_2S	Hemoglobin (Hb)	Binds and scavenges free H_2S	Prevents H_2S-mediated signaling; confirms its role in stress mitigation	Li *et al.,* 2020; Christou *et al.,* 2013

Impact of NO and H_2S on salt seedling growth attributes:

Salinity represents one of the most pervasive abiotic stressors, significantly impeding plant growth and development. Among the earliest and most pronounced effects of salt stress is the reduction in plant growth rate, primarily driven by osmotic stress arising from excessive salt

accumulation in the soil. NaCl stress has been shown to directly impact root system architecture, negatively influencing root hair formation, lateral root development, and gravitropic responses in a concentration dependent manner (Wang *et al.,* 2011; Ahanger & Agarwal, 2017). Over time, sustained exposure to high salinity also accelerates leaf senescence and reduces total photosynthetically active leaf area, ultimately diminishing photosynthetic capacity and compromising overall crop productivity.

Recent investigations have focused on the potential of gaseous signaling molecules such as H_2S and NO in enhancing plant tolerance to salinity. Exogenous application of H_2S donors, particularly NaHS, has demonstrated considerable promise in alleviating the detrimental effects of salt stress. In species such as wheat and tomato, NaHS treatment has been associated with marked improvements in key growth parameters, including FW, PH, and DW, even under saline conditions (Kaya *et al.,* 2023a; Raju & Prasad, 2023). These enhancements are attributed to the ability of H_2S to maintain ion homeostasis, stimulate antioxidant defense mechanisms, and regulate stress-responsive gene expression, collectively contributing to greater plant resilience. Likewise, the application of NO donors such as SNP has yielded similar positive outcomes. SNP has been shown to improve seedling growth traits, mitigate salt-induced oxidative damage, and support key physiological functions under NaCl stress (Kaya *et al.,* 2023a). This is primarily achieved through the regulation of antioxidant enzymes, reduction in lipid peroxidation, and improved osmotic regulation, all of which facilitate enhanced stress tolerance.

Notably, the combined application of NaHS and SNP has produced synergistic effects, further enhancing the protective outcomes observed with individual treatments. In wheat seedlings, co-application of both donors led to significantly improved FW and PH under salt stress compared to either treatment alone, suggesting a cooperative interaction between H_2S and NO signaling pathways in orchestrating plant responses to salinity (Kaya *et al.,* 2023a). This synergism indicates the possibility of integrated signaling networks that modulate growth, metabolism, and defense in response to environmental stressors. On the other hand, the application of cPTIO and L-NAME has been shown to reverse the beneficial impacts of NaHS and SNP treatments in wheat under saline conditions, thereby confirming the central role of NO in mediating the protective mechanisms initiated by these signaling molecules (Kaya *et al.,* 2023a). Collectively, these findings underscore the integrated and cooperative roles of H_2S and NO in modulating plant growth and stress adaptation under saline environments. The

mechanistic insights offered by these studies hold significant potential for developing innovative strategies aimed at improving crop resilience in salt-affected agricultural systems.

Restoration of Photosynthetic Pigments in Salt-Stressed Plants by H_2S and NO

Salinity stress significantly impairs plant physiological performance, with one of its most pronounced effects being the reduction in photosynthetic pigment biosynthesis. This decline, particularly in chlorophyll (Chl) content, is a widely accepted and sensitive indicator of cellular metabolic disruption under salt stress (Maxwell & Johnson, 2000; Chutipaijit *et al.,* 2011). Among photosynthetic pigments, chlorophyll a and b are especially susceptible to degradation in saline environments. H_2S has emerged as a potent signaling molecule with a protective role against salt-induced pigment degradation. The loss of chlorophyll under salinity is partly attributed to the activation of chlorophyll-degrading enzymes such as chlorophyllase (Gong *et al.,* 2013). However, exogenous application of H_2S donors, particularly sodium hydrosulfide (NaHS), has been shown to significantly increase chlorophyll content in stressed plants. This protective effect is largely due to H_2S ability to preserve thylakoid membrane integrity and enhance the antioxidant defense system, thereby maintaining pigment biosynthesis under saline conditions. NO similarly plays a crucial role in mitigating the deleterious effects of salinity on photosynthetic pigments. Application of NO donors has been shown to improve photosynthetic efficiency by enhancing the quantum yield of PSII and dissipating excess excitation energy, as demonstrated in *Brassica juncea* and *Lycopersicum esculentum* (Zhang *et al.,* 2011). In addition to pigment stabilization, NO facilitates the uptake and translocation of essential macro- and micronutrients such as potassium (K^+), magnesium (Mg^{2+}), zinc (Zn^{2+}), and iron (Fe^{2+}), all of which are vital for chlorophyll biosynthesis and overall photosynthetic efficiency (Dong *et al.,* 2015; Kong *et al.,* 2014; Wang *et al.,* 2013). Furthermore, NO modulates stomatal aperture by antagonizing ABA-induced closure, thus sustaining gas exchange and photosynthetic activity under salt stress (Fatma *et al.,* 2014). This NO-mediated protective role on chlorophyll-containing organelles has been observed across various crops including chickpea, rice, tomato, and eggplant (Ahmad *et al.,* 2016, 2018; Kausar & Shahbaz, 2013; Habib & Ashraf, 2014; Samiksha Singh & Prasad, 2019).

Co-treatment with sulfur and NO further augments this protective effect by restoring thylakoid membrane structure, enlarging chloroplasts, and increasing total chlorophyll content in salt-stressed *B. juncea* plants (Khan *et al.,* 2014; Fatma *et al.,* 2016). Studies conducted on rice seedlings indicated that co-treatment with H_2S and NO donors significantly increased the

number of grana lamellae and chlorophyll content, thereby safeguarding chloroplast ultrastructure and enhancing pigment stability (Mostofa *et al.,* 2015; Jiang *et al.,* 2019; Sharma & Ohri, 2024). The application of cPTIO, a selective NO scavenger, was found to reverse the beneficial effects of NO and H_2S on pigment preservation in wheat, thereby confirming NO's role in stress signal transduction (Kaya *et al.,* 2023a). Similarly, the use of L-NAME, a NOS-like enzyme inhibitor, suppressed endogenous NO production, resulting in decreased chlorophyll levels and reduced photosynthetic efficiency in mustard under salinity (Fatma *et al.,* 2016; Khan *et al.,* 2014). These findings underscore the critical function of NO biosynthesis and signaling in mitigating the impact of salinity on photosynthetic pigment integrity.

Table 2.2: Effects of H_2S and NO donors, inhibitors, and their interaction on seedling growth, photosynthetic pigments, and underlying mechanisms under salinity stress in various plant species.

Treatment	Effects on Seedling Growth	Effects on Photosynthetic Pigments	Mechanism(s)	Plant Species	References
NaCl (Salt Stress)	↓ Fresh weight, plant height, dry weight; impaired root growth	↓ Chlorophyll a, b, total chlorophyll; disrupted thylakoid structure	Osmotic stress, ion toxicity, oxidative damage, chlorophyllase activation, thylakoid damage	Wheat, Tomato, *Brassica juncea*, Rice	Wang *et al.,* 2011; Maxwell & Johnson, 2000; Ahanger & Agarwal, 2017
H_2S Donor (NaHS)	↑ Growth parameters (FW, PH, DW)	↑ Chlorophyll content; protects thylakoid membranes	Maintains ion homeostasis, enhances antioxidants, suppresses chlorophyllase, modulates stress-responsive genes	Wheat, Tomato, Rice, *Brassica juncea*	Gong *et al.,* 2013; Kaya *et al.,* 2023a

NO Donor (SNP)	↑ Growth parameters; mitigates oxidative damage	↑ Chlorophyll, improves PSII efficiency, sustains stomatal opening	Regulates antioxidant enzymes, reduces lipid peroxidation, improves nutrient uptake, modulates stomatal aperture	Wheat, *Brassica juncea*, *Lycopersicum esculentum*, Rice, Chickpea, Tomato, Eggplant	Zhang *et al.,* 2011; Fatma *et al.,* 2014
NaHS + SNP (Combined)	Synergistic enhancement in growth parameters	Synergistic increase in chlorophyll, restores chloroplast ultrastructure	Cooperative antioxidant defense, improved ion regulation, enhanced pigment biosynthesis, signaling crosstalk	Wheat, Rice, *Brassica juncea*	Mostofa *et al.,* 2015; Kaya *et al.,* 2023a
cPTIO / L-NAME	Reverses benefits of NaHS and SNP	Reverses pigment restoration	Inhibits NO signaling or biosynthesis, confirming NO's pivotal role in stress mitigation	Wheat, *Brassica juncea*	Kaya *et al.,* 2023a; Fatma *et al.,* 2016
	H_2S	Hemoglobin (Hb)	Binds and scavenges free H_2S	Prevents H_2S-mediated signaling; confirms its role in stress mitigation	Li *et al.,* 2020; Christou *et al.,* 2013

Impact of H_2S and NO on Nitrogen (N) Metabolism under Salinity

Salinity stress profoundly impairs N metabolism in plants by interfering with nutrient uptake and enzymatic functions essential for N assimilation. One of the primary consequences of salt stress is the inhibition of nitrate (NO_3^-) absorption and its subsequent reduction to nitrite (NO_2^-) and ammonium (NH_4^+), a process mediated by enzymes such as nitrate reductase (NR) and nitrite reductase (NiR). Simultaneously, salinity suppresses amino acid biosynthesis while activating hydrolyzing enzymes like RNase and proteases, which degrade existing nitrogenous compounds and further compromise plant metabolism (de Souza *et al.,* 2016; Debouba *et al.,* 2007; Nathawat *et al.,* 2005). Salt accumulation deactivates NO_3^- transporters and reduces water uptake due to altered soil water potential. Moreover, chloride ions (Cl^-) competitively inhibit NO_3^- transport across membranes, exacerbating nitrogen deficiency in plants (Balliu *et*

al., 2015; Ehlting *et al.,* 2007; Queiroz *et al.,* 2012). This competition significantly reduces total nitrogen accumulation, as reported in species such as *Solanum melongena*, *Citrus sinensis*, *Medicago sativa*, and *Triticum durum* (Abdelgadir *et al.,* 2005; Albassam *et al.,* 2001; Annunziata *et al.,* 2017; Tabatabaei *et al.,* 2006). Additionally, salinity hampers NH_4^+ uptake while promoting its intracellular accumulation, which disrupts cytoplasmic pH, disturbs osmotic equilibrium, and impairs energy metabolism, thereby intensifying stress symptoms (Dluzniewska *et al.,* 2007; Gerendás *et al.,* 1997).

Amidst these challenges, signaling molecules such as H_2S and NO have demonstrated promising roles in mitigating the adverse effects of salinity on nitrogen metabolism. The exogenous application of H_2S, particularly via NaHS, has been shown to enhance NO_3^- and NO_2^- uptake in *Lagenaria siceraria* by stimulating NR and NiR enzyme activities and upregulating the NR1 gene under salt stress (Fatima *et al.,* 2022b). H_2S also supports NiR function by modulating associated transcription factors, a process possibly facilitated by the parallel increase in endogenous NO levels. Furthermore, H_2S reduces NH_4^+ toxicity by enhancing the activities of glutamine synthetase (GS) and glutamate synthase (GOGAT), enzymes critical for the assimilation of ammonium into amino acids (Balotf *et al.,* 2018).

NO, typically delivered through SNP, also exhibits a complementary role. NO application under saline conditions improves nitrogen metabolism by upregulating NR and NiR activities and modulating the transcription of genes involved in nitrogen assimilation (Fatima *et al.,* 2022b). Additionally, NO boosts the activities of GS and GOGAT, enabling more efficient detoxification and assimilation of excess NH_4^+ into organic forms, thereby maintaining nitrogen balance and reducing stress-induced toxicity. In *Sesamum indicum* exposed to heavy metal (Pb) stress, the dual treatment significantly increased inorganic nitrogen content, indicating enhanced nitrogen metabolism through coordinated action of H_2S and NO (Huang *et al.,* 2020). This synergism is attributed to a feedback interaction between the two molecules: H_2S stimulates endogenous NO production, while NO further modulates its own biosynthetic pathways, enhancing overall efficiency. The involvement of NO in mediating these beneficial responses has been validated through the use of specific chemical inhibitors. Application of cPTIO (a selective NO scavenger) and L-NAME (a nitric oxide synthase inhibitor) partially reversed the positive effects of NaHS and SNP treatments in wheat under Cd stress. These findings underscore the critical role of NO signaling in regulating nitrogen metabolism and stress adaptation under salinity (Kaya et al. 2019; Huang *et al.,* 2020).

Impact of H_2S and NO on Mineral Ion Content under Salinity:

Salinity stress has a profound impact on plant nutrition, primarily by disrupting ion transport mechanisms, altering membrane permeability, and intensifying oxidative damage. These combined effects significantly impair the uptake, distribution, and homeostasis of essential mineral nutrients, ultimately reducing plant growth and productivity (Hänsch & Mendel, 2009; Sarwar *et al.,* 2010). One of the hallmark consequences of salinity is the excessive accumulation of sodium ions (Na^+) in plant tissues, which disturbs the delicate potassium-to-sodium (K^+/Na^+) ratio. This imbalance leads to ion toxicity, interfering with enzymatic functions, nutrient deficiencies, and in severe cases, cellular necrosis (Acosta-Motos *et al.,* 2017). To cope up with such ionic stress, plants employ adaptive strategies, including the enhancement of K^+ uptake and the restriction or compartmentalization of Na^+, particularly into vacuoles (Gadelha *et al.,* 2017). Recent studies have demonstrated the significant role of signaling molecules such as H_2S in modulating these responses. Exogenous application of H_2S has been shown to lower Na^+ concentrations while elevating K^+ levels in both root and shoot tissues. This results in an improved K^+/Na^+ ratio, which is crucial for maintaining osmotic balance and enzymatic function under saline conditions (Fatima *et al.,* 2022a; Hasanuzzaman *et al.,* 2018). In addition to ionic regulation, H_2S enhances antioxidant defenses and reduces oxidative damage to proteins, thus preserving cellular integrity and improving nutrient retention under stress.

NO, another well-established signaling molecule, similarly contributes to the regulation of ion homeostasis under salinity stress. Exogenous application of NO, typically in the form of SNP has been found to promote K^+ accumulation and restrict Na^+ influx. NO achieves this by modulating the activity and expression of key ion transporters and by strengthening antioxidant systems, thereby alleviating both ionic and oxidative stress (Matraszek *et al.,* 2016; Singh & Prasad, 2015b; Raju & Prasad, 2021a). Interestingly, the combined application of H_2S (via NaHS) and NO (via SNP) has demonstrated a synergistic effect in enhancing mineral ion balance. Studies have reported that this dual treatment more effectively reduces Na^+ accumulation and increases K^+ content in both roots and shoots than individual applications alone (Huang *et al.,* 2020). This co-treatment not only optimizes nutrient distribution but also stabilizes cellular metabolism, leading to improved stress resilience and plant performance in salt-affected environments. The application of cPTIO, a specific NO scavenger, and L-NAME, a nitric oxide synthase (NOS)-like enzyme inhibitor, partially reversed the beneficial impacts observed with NaHS and SNP treatments in tomato under Cr stress (Singh et al.2019). This

indicates that endogenous NO biosynthesis is essential for sustaining ion homeostasis under salinity (Huang *et al.,* 2020). Furthermore, these findings suggest that H_2S does not act in isolation but may stimulate or regulate NO production, thereby forming an interactive signaling network that coordinates mineral nutrient management and enhances plant tolerance to saline stress.

Table 2.3: Effects of H_2S and NO donors, individually and in combination, on nitrogen metabolism, mineral ion content, and associated mechanisms under salinity stress in various plant species.

Treatment	Effects on Nitrogen Metabolism	Effects on Mineral Ion Content	Mechanism(s)	Plant Species	References
NaCl (Salt Stress)	↓ NO_3^-, NO_2^- uptake, NR, NiR activities; ↓ amino acid biosynthesis; ↑ RNase, proteases; NH_4^+ toxicity	↑ Na^+ accumulation; ↓ K^+ content; reduced K^+/Na^+ ratio; impaired nutrient uptake	Ion toxicity, oxidative stress, competitive inhibition of NO_3^- transporters, water imbalance	*Solanum melongena, Citrus sinensis, Medicago sativa, Triticum durum*	Abdelgadir *et al.,* 2005; Tabatabaei *et al.,* 2006
H_2S Donor (NaHS)	↑ NO_3^-, NO_2^- uptake; ↑ NR, NiR, GS, GOGAT activities; ↓ NH_4^+ toxicity	↓ Na^+, ↑ K^+, improved K^+/Na^+ ratio	Stimulates nitrogen assimilation enzymes, enhances ion homeostasis, modulates transcription factors	*Lagenaria siceraria,* Wheat, Tomato	Fatima *et al.,* 2022b; Hasanuzzaman *et al.,* 2018
NO Donor (SNP)	↑ NR, NiR, GS, GOGAT activities; improved NH_4^+ assimilation	↓ Na^+, ↑ K^+ uptake, improves ion balance	Modulates ion transporter expression, enhances antioxidant enzymes, regulates nitrogen assimilation	Wheat, Tomato, *Sesamum indicum*	Fatima *et al.,* 2022b; Singh & Prasad, 2015b
NaHS + SNP (Combined)	Synergistic increase in NO_3^-, NO_2^-, NR, NiR, GS, GOGAT activities; ↓ NH_4^+ toxicity	More effective than individual treatments in lowering Na^+ and boosting K^+ content	Cross-talk between NO and H_2S signaling pathways, cooperative upregulation of ion transporters and enzymes	Wheat, Rice, Tomato, *Sesamum indicum*	Huang *et al.,* 2020; Raju & Prasad, 2021a

Role of H_2S and NO in Modulating ROS and Oxidative Stress Under Salinity:

Elevated salt concentrations reduce the cellular water potential, triggering stomatal closure and limiting CO_2 assimilation. These physiological disturbances disrupt the electron transport chain leading to the excessive generation of reactive oxygen species (ROS), including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$) (Ahmad *et al.,* 2010, 2011, 2016; Krieger-Liszkay *et al.,* 2005). At high levels, ROS become toxic, damaging vital biomolecules such as proteins, lipids, and nucleic acids, thereby impairing metabolic functions and triggering programmed cell death (Apel & Hirt, 2004; Asada, 2006; Mittler *et al.,* 2011). Numerous studies have recorded elevated H_2O_2 content and enhanced lipid peroxidation in salt-stressed cultivars of wheat (*Triticum aestivum*), mustard (*Brassica juncea*), rice (*Oryza sativa*), citrus (*Citrus aurantium*), tomato (*Solanum lycopersicum*), and eggplant (*Solanum melongena*) with salt-sensitive genotypes exhibiting more pronounced damage than tolerant ones (Parida & Das, 2005; Sairam *et al.,* 2002; Parihar *et al.,* 2015b; Hasanuzzaman *et al.,* 2011; Ahmad *et al.,* 2016).

To counter these oxidative effects, plants activate a sophisticated antioxidant defense system, often regulated or enhanced by gaseous signaling molecules such as hydrogen sulfide (H_2S) and nitric oxide (NO). Exogenously applied H_2S, most commonly in the form of NaHS, has been shown to upregulate antioxidant enzymes such as SOD, CAT, and APX, thereby enhancing ROS detoxification and restoring redox homeostasis under salinity (Hasanuzzaman *et al.,* 2018; Wang *et al.,* 2023; Raju & Prasad, 2021). For instance, in salt-stressed mungbean and pepper, NaHS treatment reduced MDA content and preserved chlorophyll and membrane integrity (Guo *et al.,* 2018; Wang *et al.,* 2023). Likewise, in tomato and strawberry, H_2S has been shown to maintain antioxidant capacity and limit oxidative damage through stabilization of cellular membranes and prevention of lipid peroxidation (Christou *et al.,* 2013; Mishra *et al.,* 2024). Similarly, NO, applied as SNP, has emerged as a potent modulator of antioxidant defense. SNP treatment enhances enzymatic activity (e.g., SOD, CAT, GR, APX), improves the AsA-GSH cycle efficiency, and reduces electrolyte leakage under salt stress (Johnson *et al.,* 2009; Farnese *et al.,* 2017; Hasanuzzaman *et al.,* 2020). For example, in wheat, chickpea (*Cicer arietinum*), and rice, SNP mitigated salinity-induced oxidative damage by enhancing both enzymatic and non-enzymatic antioxidants (Ahmad *et al.,* 2016; Sharma *et al.,* 2020). SNP has also demonstrated effectiveness in alleviating heavy metal and salt stress. In *Zea mays* under chromium toxicity and in lettuce (*Lactuca sativa*) exposed to salinity, NO treatment reduced hydrogen peroxide (H_2O_2) accumulation and activated mitogen-activated protein kinase (MAPK) signaling pathways, thereby enhancing stress tolerance (Egbichi *et al.,* 2014;

Kharbech *et al.,* 2022).Moreover, the co-application of H_2S and NO exhibits a synergistic effect in reinforcing plant tolerance to salt-induced oxidative stress. In *Fragaria* × *ananassa*, dual treatment with NaHS and SNP reduced oxidative markers (H_2O_2, MDA) and increased the activity of antioxidant enzymes more efficiently than individual treatments (Christou *et al.,* 2013). Similar synergistic enhancements have been reported in cucumber, rice, and *Brassica campestris*, where combined treatment significantly improved APX and GR activity, maintained ion homeostasis, and improved growth and stress resilience under salinity (Bhuyan *et al.,* 2020; Jiang *et al.,* 2019; Mishra *et al.,* 2024). Application of cPTIO (an NO scavenger) and L-NAME (a nitric oxide synthase inhibitor) effectively reversed the protective effects of both SNP and NaHS, leading to decreased antioxidant enzyme activity, elevated H_2O_2 levels, and increased cellular damage under salinity and arsenic stress in tomato, rice, and brinjal (Suhel, 2023; Kaya *et al.,* 2023; Huang *et al.,* 2020). These results underline that while both H_2S and NO individually contribute to antioxidant regulation, their synergistic action and interdependent signaling are essential for efficient oxidative stress mitigation and improved plant adaptation to saline environments.

Impact of H_2S and NO on antioxidants under salinity:

Excess salt concentrations reduce water uptake, disrupt nutrient acquisition, and accelerate the accumulation of ROS such as O_2^-, H_2O_2, •OH, and $^1O_2$. These ROS inflict oxidative damage on cellular structures including lipids, proteins, and DNA, resulting in impaired metabolism, membrane peroxidation, and growth inhibition (Apel & Hirt, 2004; Mittler *et al.,* 2011). H_2S, applied exogenously as NaHS, functions both as a direct antioxidant and a modulator of antioxidant gene expression. It enhances the activities of enzymes such as SOD, CAT, APX, GR, and GST, thereby maintaining redox homeostasis and protecting plants from ROS-induced injury (Hasanuzzaman *et al.,* 2018; Raju & Prasad, 2021). In *Arabidopsis thaliana*, NaHS treatment upregulated GPX activity under methyl-viologen-induced oxidative stress (Ozfidan-Konakci *et al.,* 2023). Similarly, in tomato and brinjal seedlings, exogenous H_2S improved antioxidant capacity and reduced malondialdehyde (MDA) levels under NaCl stress (Raju & Prasad, 2021). In strawberry leaves, H_2S mitigated salt-induced oxidative damage by enhancing the activity of antioxidant enzymes (Christou *et al.,* 2013). Similarly, in alfalfa plants, exposure to NaCl stress led to an increase in endogenous H_2S levels, indicating that H_2S may function as a secondary messenger in plant stress signaling (Lai *et al.,* 2014). In rice, combined application of H_2S and NaCl enhanced seedling growth and decreased ROS and MDA accumulation, affirming its protective role under salinity (Mishra *et al.,* 2024). A recent study

revealed that melatonin enhances drought tolerance in bread wheat by promoting H_2S production, which activates antioxidant defenses and alleviates oxidative stress. Inhibition of H_2S synthesis reversed these effects, highlighting its essential role in melatonin-induced antioxidant regulation (Muslemyar & Kaya, 2025).

NO is another crucial signaling molecule with established roles in oxidative stress mitigation. NO directly scavenges ROS and regulates the expression of stress-responsive genes via pathways such as mitogen-activated protein kinase (MAPK). Exogenous application of NO donors like SNP has been shown to significantly enhance the antioxidant enzyme activities of SOD, CAT, APX, and GR in a wide range of crops. In cucumber (*Cucumis sativus*), SNP treatment under salinity improved enzymatic antioxidant activity and reduced MDA content, electrolyte leakage, and $O_2^{\cdot-}$ production (Fan *et al.,* 2013). In chickpea (*Cicer arietinum*), 0.2 mM SNP significantly increased antioxidant enzymes while lowering ROS-induced damage (Sheokand *et al.,* 2008). In *Triticum aestivum*, *Zea mays*, and *Pisum sativum*, SNP-induced tolerance was linked to NO-mediated regulation of ROS metabolism and antioxidant defenses (Ahmad *et al.,* 2016; Kaur *et al.,* 2018; Yadu *et al.,* 2017). Moreover, the use of NO-releasing compounds like DETA and SNAP improved salinity tolerance in chickpea by increasing the activities of SOD, CAT, APX, and GR, while decreasing H_2O_2 and MDA levels (Ahmad *et al.,* 2016; Egbichi *et al.,* 2014). Nitric oxide (NO) seed priming has shown promising effects in enhancing plant stress tolerance. In *Citrus aurantium*, pretreatment of roots with SNP (a NO donor) significantly increased the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase (CAT) (Ahanger *et al.,* 2017). Similarly, in *Oryza sativa* seedlings, NO application enhanced overall antioxidant capacity and conferred greater resistance to stress conditions (Sharma *et al.,* 2020; Uchida *et al.,* 2002). In banana and mustard, SNP application significantly improved antioxidant responses and reduced oxidative injury under osmotic and salinity stress (Muhammad *et al.,* 2021; Sharma *et al.,* 2020).

Interestingly, co-application of H_2S and NO has demonstrated synergistic effects in enhancing stress resilience. In *Fragaria* × *ananassa* (strawberry), the combined treatment with NaHS and SNP more effectively reduced oxidative markers (H_2O_2, MDA) and improved antioxidative enzyme activities compared to individual treatments (Christou *et al.,* 2013). In rice seedlings, co-treatment improved SOD activity and preserved growth and cellular integrity under salt and heat stress (Mishra *et al.,* 2024). Likewise, studies in maize revealed that NO stimulates endogenous H_2S production under hypoxic stress, which in turn enhances antioxidant defenses,

suggesting a feedback mechanism between these two molecules (Gong *et al.,* 2020). In sesame seedlings, the combined treatment under heavy metal stress led to increased inorganic nitrogen levels and higher GST activity, reinforcing the interactive role of H_2S and NO in stress signaling (Huang *et al.,* 2020).

To substantiate the involvement of NO in these protective mechanisms, specific inhibitors and scavengers such as cPTIO (a NO-specific scavenger) and L-NAME (a nitric oxide synthase inhibitor) have been widely used. Their application reversed the stress-alleviating effects of NO and H_2S treatments in multiple studies. For instance, Suhel (2023) demonstrated that L-NAME and cPTIO negated SNP-induced antioxidant benefits in tomato and brinjal seedlings under arsenic toxicity. In cucumber, these inhibitors reduced GST activity and elevated oxidative stress markers (Kaya *et al.,* 2023). Such findings underscore the essential role of NO signaling in coordinating the antioxidant response under saline conditions, often in cooperation with H_2S signaling.

Impact of H_2S and NO on enzymes and metabolites of Ascorbate glutathione cycle under salinity:

Under salinity stress, plants experience heightened levels of hydrogen peroxide (H_2O_2), necessitating efficient detoxification through the ascorbate-glutathione (AsA-GSH) cycle. H_2S, applied exogenously as NaHS, plays a vital role in upregulating key enzymatic antioxidants of this cycle: ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR), and dehydroascorbate reductase (DHAR). Studies on wheat and tomato have shown that NaHS treatment under salt stress significantly increases the activity of APX and GR, thereby promoting H_2O_2 detoxification and maintaining chloroplast stability (Raju & Prasad, 2021; Mishra *et al.,* 2024). In *Arabidopsis thaliana*, NaHS treatment has been reported to boost glutathione peroxidase (GPX) activity under oxidative stress, further enhancing cellular redox homeostasis (Ozfidan-Konakci *et al.,* 2023). Similarly, nitric oxide (NO) enhances the AsA-GSH cycle through SNP application by elevating APX, GR, and DHAR activities. For instance, in *Triticum aestivum* seedlings exposed to 300 mM NaCl, SNP treatment improved the antioxidant status by increasing DHAR and GR levels along with non-enzymatic antioxidants like GSH and AsA (Hasanuzzaman & Fujita, 2011; Hasanuzzaman *et al.,* 2020). Furthermore, NO has been shown to modulate MDHAR activity in chickpea and mustard, contributing to rapid regeneration of AsA and strengthening antioxidant defense (Ahmad *et al.,* 2016; Sharma *et al.,* 2020). Notably, co-application of H_2S and NO results in a

synergistic enhancement of AsA-GSH cycle enzyme activities. In *Brassica campestris* and cucumber, combined treatment with NaHS and SNP led to significantly higher APX and GR activities compared to individual treatments, ensuring more efficient H_2O_2 scavenging and reduced oxidative damage (Bhuyan *et al.,* 2020; Zeeshan *et al.,* 2020). This cooperative effect of the two molecules further improved stress tolerance by accelerating the recycling of AsA and GSH. However, the critical involvement of NO in this process has been confirmed through inhibitor studies. The use of cPTIO, a NO-specific scavenger, and L-NAME, a nitric oxide synthase inhibitor, effectively reversed the beneficial effects of H_2S and NO on AsA-GSH cycle enzyme activities. For example, in SNP- and NaHS-treated tomato and brinjal seedlings, cPTIO and L-NAME application led to a marked reduction in APX and GR activities and elevated H_2O_2 accumulation under salinity and arsenic stress (Suhel, 2023; Kaya *et al.,* 2023). These findings confirm that H_2S and NO individually and synergistically activate the AsA-GSH cycle, and that NO signaling is essential for sustaining this antioxidant defense under salinity stress.

Table 2.4: Effects of exogenous H_2S and NO treatments on antioxidant defense systems ROS detoxification, and AsA-GSH cycle enzyme activities in various plant species under salinity stress. The table also highlights the synergistic effects of combined H_2S and NO applications, and the reversal of protective effects by cPTIO and L-NAME

Treatment	Plant Species	Effects on Antioxidant Enzymes	Effects on ROS & Oxidative Damage	Effects on AsA-GSH Cycle Enzymes	Key References
H_2S (NaHS)	Mungbean, Pepper, Tomato, Strawberry, *Arabidopsis*, Wheat, Brinjal, *Alfalfa*, Rice	↑ SOD, CAT, APX, GR, GST, GPX, enhanced chlorophyll stability	↓ H_2O_2, MDA, lipid peroxidation, membrane damage	↑ APX, GR, MDHAR, DHAR, GPX activity, improved H_2O_2 detoxification	Hasanuzzaman *et al.,* 2018; Raju & Prasad, 2021; Mishra *et al.,* 2024; Christou *et al.,* 2013; Ozfidan-Konakci *et al.,* 2023
NO (SNP)	Wheat, Chickpea, Rice, Cucumber, Citrus, Banana, Mustard, *Zea mays, Pisum sativum*	↑ SOD, CAT, APX, GR, GST, enhanced antioxidant gene expression	↓ H_2O_2, MDA, electrolyte leakage, improved membrane integrity	↑ APX, GR, DHAR, MDHAR, GSH, AsA, improved AsA-GSH cycle efficiency	Ahmad *et al.,* 2016; Sharma *et al.,* 2020; Hasanuzzaman *et al.,* 2020; Fan *et al.,* 2013; Muhammad *et al.,* 2021
H_2S + NO (NaHS + SNP)	Strawberry, Rice, Cucumber, *Brassica campestris, Maize, Sesame*	Synergistic ↑ in SOD, CAT, APX, GR, GST, stabilized photosynthesis	Greater ↓ in H_2O_2, MDA, improved chlorophyll, membrane stability	Synergistic ↑ in APX, GR, MDHAR, DHAR, rapid AsA & GSH recycling	Christou *et al.,* 2013; Bhuyan *et al.,* 2020; Mishra *et al.,* 2024; Gong *et al.,* 2020; Huang *et al.,* 2020

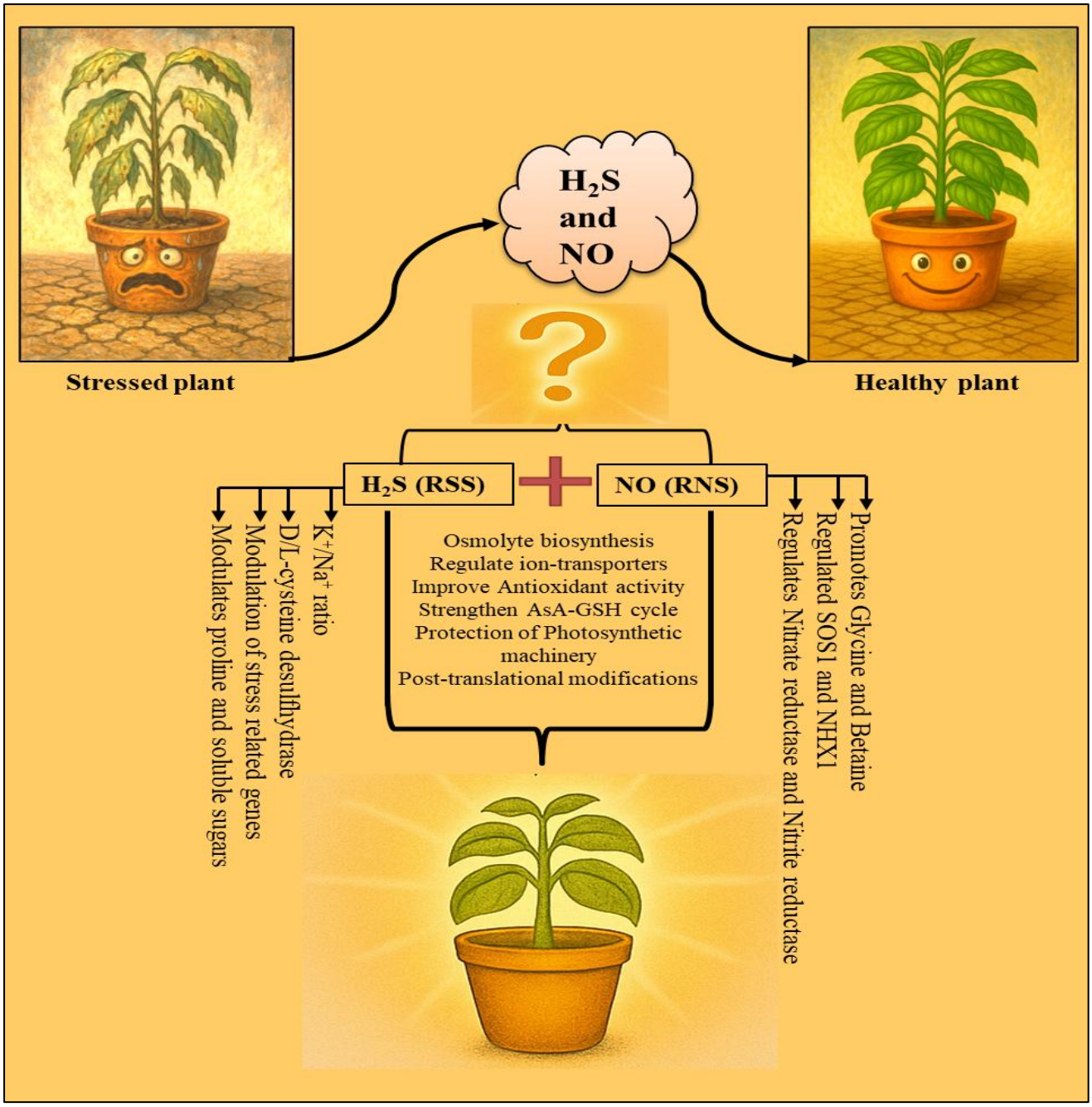


Figure 2.1: Visual Representation of Plant Stress Recovery: The Role of H_2S and NO Signaling in Resilience.

CHAPTER 3

MATERIAL AND METHODS

3.1 Experimental plant:

Cucumber (*Cucumis sativa*) and bitter gourd (*Momordica charantia*) are cultivated for their fruits and are technically called pepo, a type of berry with a hard outer rind. These are annual creeping vines that climb using tendrils. Both these are indigenous vegetables thriving in tropical regions of Asia and taxonomically belong to the Cucurbitaceae family.

Kingdom: Plantae
Division (Phylum): Angiosperms
Class: Dicotyledons
Order: Cucurbitales
Family: Cucurbitaceae
Genus: *Cucumis*
Species: *sativus*

Figure 3.1: Taxonomy of Cucumber plant (*Cucumis sativus*) showing developing fruits and flowers on a vining, ground-level growth habit.

Kingdom: Plantae
Division (Phylum): Angiosperms
Class: Dicotyledons
Order: Cucurbitales
Family: Cucurbitaceae
Genus: *Momordica*
Species: *charantia*

Figure 3.2: Taxonomy of Bitter gourd plant (*Momordica charantia*) showing developing fruits and flowers on a vine.

3.2 Seed and growth conditions

The cucumber, and bitter gourd seeds were bought from Durga Seed Farm, Chandigarh. The seeds were thoroughly washed, surface sterilized for 5 minutes in a 2% (v/v) NaClO and soaked in distilled water for 2-4 hours. For germination, sterilized seeds were placed in the dark after being wrapped in muslin cloth. After this, germinated seeds were planted in a tray filled with sterilized sand, and this was followed by an incubation of the seeds in the dark at 25 ± 2°C for 48 h. After that, trays were moved into a growth chamber with a PAR of 250 mol photons per square meter per second, 16:8-hour day/night phase, and 65–70% relative humidity until the emergence of secondary leaves. After carefully removing the sand adhered to the roots, seedlings were acclimatized with a half-strength Hoagland medium with a pH of 7.0 for 24 hours (Hoagland and Arnon,1950). The doses of NaCl (25 mM), SNP (90 µM) and NaHS (40 µM) were chosen from our past preliminary screening investigations.

3.2.1 Nutrient media:

Seedlings were cultivated using a nutrient medium derived from half-strength Hoagland and Arnon's formulation (Hoagland and Arnon's, 1950), which incorporated both micronutrients (mgL^{-1}) and macronutrients (gL^{-1}). Analytical grade (AR) chemicals and solvents utilized in the research were acquired from Sigma Aldrich, Loba Chemei, Himedia, Merck, and CHD etc.

3.2.2 Glassware and chemicals:

The glassware used in this study, including beakers, flasks, petri plates, pipettes, and test tubes were exclusively sourced from 'borosil' to ensure high-quality materials. Analytical grade (AR) chemicals and solvents utilized in the research were acquired from reputable suppliers such as Sigma Aldrich, Loba Chemei, Himedia, Merck, and CHD etc. Additionally, fine biochemical-grade chemicals were obtained from E-Merck, Germany, and Sigma Chemical Co. USA. To uphold the uniformity and reproducibility of results, all reagents and standards were meticulously prepared using DDW. This stringent approach to sourcing glassware and chemicals contributes to the reliability and precision of the experimental outcomes.

3.2.3 Sanitization of glassware, nutrient media, and seeds:

All glassware and the Hoagland media underwent sterilization in an autoclave for 15 minutes at a pressure of 15 lbs $inch^{-2}$ and at 121ºC. Conversely, plastic cups and trays used in this study were precisely rinsed and subjected to surface sterilization using absolute alcohol.

Seedlings were cultivated in sand and methodically washed multiple times with tap water. Subsequently, concentrated HCl was added to dissolve any organic matter present. After a 24-hour, the sand was thoroughly washed with water and then by DDW. The acid-washed sand was then dried and sterilized at 250 ºC for two hours.

The healthy and uniform seeds of the cucumber and bitter gourd were sterilized using 2% (v/v) NaClO for 15 minutes. The sterilized seeds were then subjected to multiple rinses with DDW to eliminate any residual sterilizing agents.

3.2.4. NaCl treatment:

In this study, sodium chloride (NaCl) was chosen for salt treatment. NaCl (molecular mass); 58.44g mol^{-1}, purity=98%; Merck, Mumbai, India) was used in different concentrations: 5, 10, 15, 20, 25, 30, 35, 40, 45, and 50 mM. the solutions were prepared in 50ml Hoagland medium and applied to seedlings with secondary leaves grown under specified conditions. The electrical conductivity of the given solution was adjusted every two days until the seventh day of treatment because various crops can endure differing salinity content in irrigation water. The electric conductivity (E.C.) for a salt solution that ranges from 3.28 to 3.6 dS m-1 (Deci Siemens per meter), has been adjusted for the present study. According to study given by Fipps (2003) from the Texas A&M Agrilife Extension Service, the permissible limit of NaCl in irrigation water is between 9-24mM, though the salt tolerance index varies among different crops. The salinity level in irrigation water is influenced by multiple factors, ranging from 0.6-3.1 dS/m (approximately 6.6-34mM) for crops (George, 1983). Fresh mass and plant height were measured after seven days of treatment. Based on screening experiments, a concentration of 25mM was selected for this study and this concentration is environment realistic.

3.2.5. Source, preparation and mode of signalling molecules, inhibitors and scavenger treatment:

In this study, different concentrations of SNP (Donor of NO; molecular mass=297.95g/mol), and NaHS (Donor of H_2S; Molecular mass= 56.07 g/mol) were chosen for salt (NaCl) treatments in the study. NaHS at various doses of 0, 10, 20, 30, 40, 50, 60, 60, 70, and 80 µM, and SNP at various doses of 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 µM were used for screening. Based on screening experiments, the selected doses were SNP (90 µM), and NaHS (40 µM) that were prepared in 50ml Hoagland medium. 100 µM of cPTIO, 100 µM of L-NAME were chosen from the literature survey.

3.2.6. Treatment design:

Following screening experiments, test seedlings were placed in a growth medium containing sodium chloride (NaCl) 25 mM, 40 µM of sodium hydrogen sulfide (NaHS), 90 µM of sodium nitroprusside (SNP), 100 µM of cPTIO and 100 µM of L-NAME. The experimental design included several combinations which were selected based on Kaya *et al.,* (2023), Singh *et al.,* (2019): (1) control (without treatment), (2) NaCl, (3) NaCl + NaHS, (4) NaCl + SNP, (5) NaCl

+ NaHS+ SNP, (6) NaCl + NaHS + L-NAME, (7) NaCl + SNP + L-NAME, (8) NaCl + NaHS + cPTIO and (9) NaCl + SNP + cPTIO. Each combination was tested with three replicates, and the trials were performed thricely and separately. After seven days, seedlings were harvested, and various parameters were analyzed. The experimental design is presented in the form of flow chart **(Figure 3.1).**

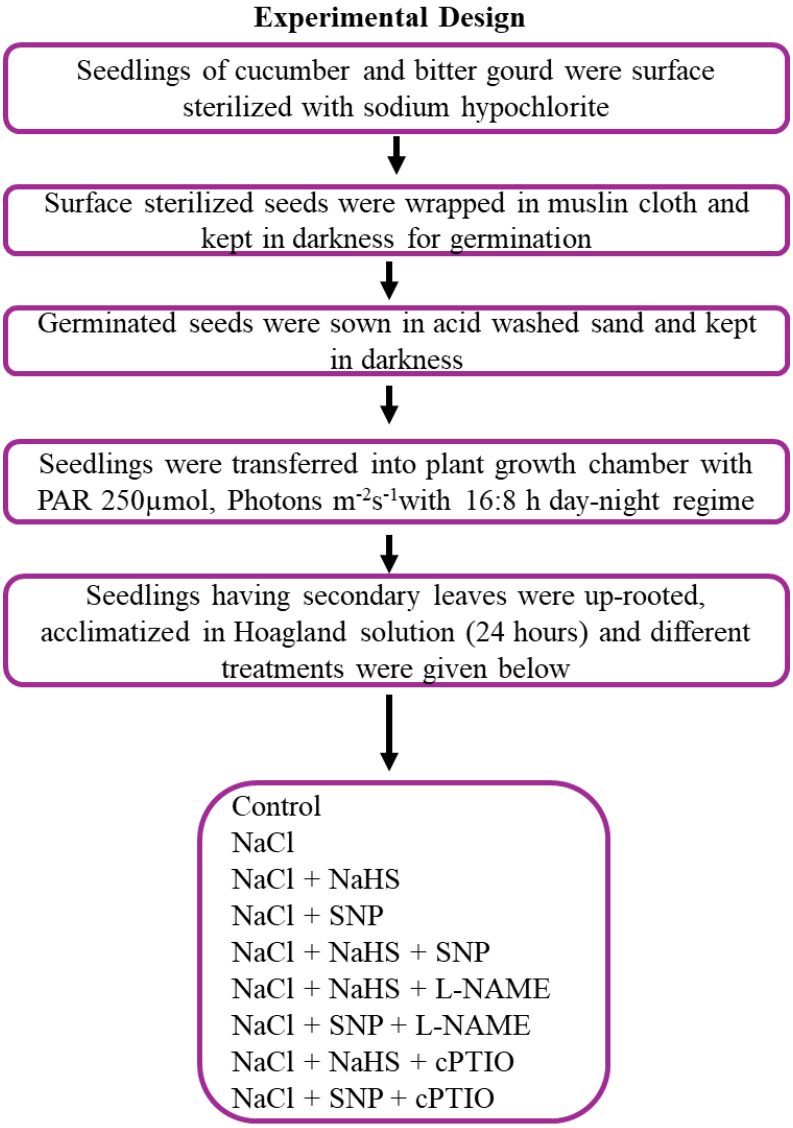


Figure 3.3: The flow chart depicts the overview of the experimental strategy

3.3 **Growth behaviour of test seedlings:**

3.3.1 Growth attributes

Growth parameters in terms of PH, FM, and DM were recorded. The FM and PH were recorded at the time of sampling whereas for DM, the seedlings were oven dried at a temperature of 80℃ for 48 hours.

3.3.2 Photosynthetic pigment contents: chlorophyll and carotenoids

The content of different fractions (Chl a and Chl b) was estimated from harvested samples according to (Lichtenthaler, 1987). Fresh samples were crushed thoroughly with 80% acetone and centrifuged at 10, 000 g for 10 min at 4°C. The absorbance was observed at 663.2, 646.8 and 470 nm spectrophotometerically. The quantity of chlorophylls and carotenoid contents were calculated using the following equations

Chl a: $Ca = 12.25A_{663.2} - 2.79A_{646.8}$ (µg/ml)

Chl a: $Cb = 21.50A_{646.8} - 5.10A_{663.2}$ (µg/ml)

Total Carotenoids: $Cxc\ (1000A_{470} - 1.82Ca - 85.02Cb) / 198$ (µg/ml)

3.4 Determination of H_2S and NO content

(a) H_2S content:

Methodology: Mostofa *et al.,* (2015)

	Reagent	**Concentration**
Homogenization	Potassium-Phosphate Buffer (pH 7.0)	100 mM
	EDTA	10 mM
	Extraction Buffer	
Reaction	5'-Dithiobis (2-nitrobenzoic acid) (DTNB)	20 mM

Homogenize 250mg of leaf sample in a mixture, centrifuge the whole content at 11,200×g for 15minutes at 4°C. Thereafter, mix 100μL of supernatant with 1,880 μL of extraction buffer and make a final volume of 2ml. Now was incubated at 25 °C for 5 minutes and then take a reading at 412nm. Finally, quantify H_2S by using a standard prepared by known concentration of NaHS.

(b) NO content:

Methodology: (Zhou *et al.,* 2005).

	Reagent	**Concentration**
Homogenization	Acetate Buffer (cool)	50 mM
Supernatant Treatment	Charcoal	0.25 mg
Reaction	Griess Reagent	1%

Leaves (250mg) were homogenized and centrifuged at 4°C for 15min at 10,000g. After that, supernatant was collected, and charcoal was added to neutralize the sample. Again, the samples were centrifuged for 20min. and 1ml of supernatant was taken and add 1ml of 1% Griess reagent to it. A pink colour was observed, and absorbance was measured at 540nm using spectrophotometer.

3.5 Determination of inorganic nitrogen content:

Leaf samples (1g) were crushed in 10ml of DW, then filtered using Whatman no. 1 filter paper. The resulting filtrate was used to estimate nitrate, nitrite, and ammonium contents.

3.5.1 Estimation of nitrate (NO_3^-) content:

Methodology: Cataldo *et al.,* (1975)

	Reagent	**Concentration**
Enzyme Extract	Salicylic Acid (in conc. H_2SO_4)	0.1 ml 5%
Neutralization	NaOH	4 M

The reaction mixture consists of was prepared in conc. H_2SO_4 and was incubated at room temperature for 15 minutes and then add 1ml NaOH (4M). After cooling at normal temperature, absorbance was measured at 410nm, and nitrate content was calculated by calibration curve prepared with KNO_3.

3.5.2 Estimation of nitrite (NO_2^-) content:

Methodology: Snell and Snell (1949)

	Reagent	Concentration
Reaction Mixture	Tissue Extract	0.1 ml
	Distilled Water (DW)	0.9 ml
	Sulphanilamide	1 ml
	NEDD	1 ml

Reaction mixture contained 0.1ml of tissue extract, 0.9ml of DW, 1ml of sulphanilamide and 1ml of NEDD. The pink colour had appeared after 10min, was measured at 540nm and nitrite content was calculated by using standard curve prepared by KNO_2.

3.5.3 Estimation of ammonia (NH_4^+) content:

Methodology: Molins-Legua *et al.,* (2006)

	Reagent	Concentration
Reaction Mixture	Tissue Extract (Filtrate)	0.1 ml
	Na^+-K^+ Tartrate	0.01 ml
	Double Distilled Water	2.4 ml
	Nessler's Reagent	0.1 ml

The reaction mixture consisted of 0.1ml tissue extract (filtrate), 0.01ml Na^+-K^+ tartrate, 2.4ml double distilled water (DDW) and 0.1 ml Nessler's reagent. After 5min, the absorbance was recorded at 425nm, and ammonia content was calculated by calibration curve prepared by NH_4Cl.

3.5.4 Enzymes of nitrate assimilation

3.5.4.1 Nitrate reductase (NR) activity:

Methodology: Hayaishi *et al.,* (2005)

	Reagent	Concentration
Reaction Mixture	Phosphate Buffer (pH 7.5) KNO_3	0.1 M 0.02 M
Diazotization Reaction	Sulphanilamide (in 3M HCl) NEDD	1% (0.3 ml) 0.02% (0.4 ml)

Samples (100mg) were crushed ice-cold mortar using 5ml of 0.1M phosphate buffer (pH 7.5), then centrifuge. Add 0.02M KNO_3 and kept in dark for 30 minutes at 25°C. Nitrite was formed after the diazotization with each of 0.3 ml of 1% sulphanilamide in 3M HCl and 0.02% NEDD in 0.4ml of enzyme extract. After twenty minutes of incubation the reaction was stopped by adding 1ml of DW and optical density was measured at 540nm which was calculated by the standard curve prepared by $NaNO_2$.

3.5.4.2 Nitrite Reductase (NiR) activity:

Methodology: Losada and Paneque, (1971)

	Reagent	Concentration/Volume
Preparation of homogenate	Potassium phosphate buffer	0.1 M, pH 8.0
Reaction Mixture	Tris buffer	1 ml
	Sodium nitrite	0.2 ml
	Methyl viologen	0.3 ml
	Enzyme extract	0.02 ml
	Sodium dithionite	0.3 ml
	Final volume	2 ml

An ice–cold mortar with 250 mg fresh leaves were standardized using 0.1 M potassium phosphate buffer (pH 8.0). The homogenate was centrifuged at 15,000 g for 20 min and the supernatant was used as enzyme extract for NiR activity. The reaction mixture contains the reagent above given in the table. Incubate for 10 min. at 30°C, after it the reaction was completed, vortex the mixture for a minute and read at 540 nm note the reading and expressed in terms of μmol NO_2^- reduced g^{-1} FM h^{-1}.

3.5.5 Determination of ammonia assimilating enzymes: GS-GOGAT pathway

3.5.5.1 Glutamine synthetase (GS) activity:

Methodology: Cthrine Lillo, (1984)

	Reagent	Concentration
Extraction Medium	Tris-HCl (pH 7.8)	200 mM
	Glycerol	15% (v/v)
	2-Mercaptoethanol	14 mM
	EDTA	1 mM
	Triton X-100	0.1% (v/v)
Reaction Mixture	L-Glutamate	500 mM
	ATP	100 mM
	$MgSO_4$	300 mM
	$NH_2OH.HCl$ (neutralized with NaOH)	200 mM
	Tris-HCl (pH 8.0)	200 mM
Reaction Termination	$FeCl_3$	2.5% (w/v)
	TCA (in 1.5 M HCl)	5% (w/v)

Fresh samples (100 mg) homogenized in an extraction medium containing given regent in the table, kept cool during the process. The homogenate was then centrifuged for 15 minutes at 10,000 g at 4°C. The reaction mixture (2 ml), with the pH adjusted to 8.0. After adding 0.2 ml of the enzyme extract, the reaction was initiated and place for 30 minutes at 25°C. The reaction was terminated by adding 4 ml of the given reagents. The reading was measured at 540 nm, and the activity was stated as A_{540} nm g^{-1} FM h^{-1}.

3.5.3.2. Glutamate synthase (GOGAT) activity:

Methodology: Singh and Srivastava, (1986).

	Reagent	Concentration
Extraction Medium	Sodium phosphate buffer (pH 7.5)	0.2 M
	EDTA	2 mM
	KCl	50 mM
	2-Mercaptoethanol	0.1% (v/v)
	Triton X-100	0.5% (v/v)
Reaction Mixture	Sodium phosphate buffer (pH 7.3)	25 mM
	EDTA	1 mM
	L-Glutamine	20 mM
	2-Oxoglutarate	5 mM
	KCl	50 mM
	NADH	1 mM

The 100 mg fresh samples were homogenized in extraction medium which consisted of given reagent under cool condition. The homogenate was centrifugated at 10, 000 g for 15 min at 4 °C. Add L-glutamine, in the reaction mixture and the reaction was started and reduction in the absorbance was noted spectrophotometrically at 340 nm for 5 min. The enzyme activity was determined using a standard curve prepared with NADH and was expressed in terms of nmol NADH oxidized g^{-1} FM h^{-1}.

3.6 Estimation of Na^+ and K^+ content:

Sodium (Na^+), potassium (K^+), contents in untreated and treated samples were estimated by the method of Kaur *et al.,* (2023). Leaf samples were first oven-dried at 80℃ for 48h, 100mg of dried and grounded sample was put in a digestion vessel and 10ml of concentrated HNO_3 was added. This sample was left overnight at room temperature. Next day, the sample was heated until NO_2 fumes production vanished and then the beaker was allowed to cool. After this, 3ml of 70% $HClO_4$ was added, heated again until a small volume was left, then transferred the sample to a flask and diluted it with distilled water. Thereafter, the solution was passed through medium filters into plastic bottles. Then Na^+ and K^+ content was measured by using an atomic absorption spectrometer by calibrating it with a standard solution of Na^+ and K^+. Finally, the concentration of Na^+ and K^+ was determined by using standard conditions.

3.7 Total soluble protein content:

Methodology: LOWRY *et al.,* (1951)

Reagent	Concentration/Volume
BSA Stock Solution	1 mg/ml
Analytical Reagents a) Solution A (Sodium carbonate) b) Solution B (Copper sulphate) c) Solution C (Mix of A and B)	2% in 0.1N NaOH 0.5% in 1% potassium tartrate 50 ml of Solution A + 1 ml of Solution B
2N FCR (Folin-Ciocalteu Reagent)	2N in 0.1N NaOH
Tris buffer	pH 7.0

BSA solutions of varying concentrations (40-200 µg) were prepared by diluting the BSA stock solution (1 mg ml^{-1}) with distilled water. For each test tube, 2 ml of analytical reagent was added, and the contents were thoroughly mixed. For 10 minutes, the tubes were kept at room temperature, after which the addition of 0.2 ml of Folin-Ciocalteu Reagent (FCR) was done. The mixtures were then incubated at 37°C for 30 minutes. The optical density (O.D.) of the samples was estimated at 660 nm spectrophotometerically. A standard calibration curve was created by plotting absorbance values against the corresponding BSA concentrations. The absorbance of the test samples was measured and calculated using the standard curve.

3.8 Determination of oxidative stress biomarkers (SOR, H_2O_2 and MDA):

Estimation of reactive oxygen species:

Fresh seedling samples (0.5 g) of Cucumber and bitter gourd were homogenized in 0.1% TCA and centrifuge it for 15min. at 10,000×g at 4°C and the resulting supernatant was used for the estimation of oxidative stress biomarkers.

3.8.1 H_2O_2content:

Methodology: Alexieva *et al.,* (2001)

	Reagent	Concentration/Volume
Reaction Mixture	Enzyme extract 50 mM Tris-HCl buffer (pH 7.0) 1 M KI reagent	0.5 ml 0.5 ml 2 ml

Tubes containing the reaction mixture were incubated for 1 h in darkness and the absorbance was recorded at 390 nm and determined by a standard curve and expressed as μM (g FW)$^{-1}$. s

3.8.2 Superoxide anion content ($O_2^{\cdot-}$):

Methodology: Elstner and Heupel (1976)

	Reagent	Concentration/Volume
Reaction mixture	50 mM Tris-HCl buffer (pH 7.0) 10 mM Hydroxylamine hydrochloride Enzyme extract	0.9 ml 0.1 ml 240 μg
Addition of reagents	17 mM Sulphanilamide 7 mM α-Naphthylamine (prepared in 50% ethanol)	Add to the reaction mixture Add to the reaction mixture

Experiment involved incubating reaction mixture and 240 μg of enzyme extract for 30 minutes at 25°C. In the first step, O_2^- reacts with hydroxylamine hydrochloride to form nitrite. Next, 17 mM sulphanilamide and 7 mM α-naphthylamine (prepared in 50% ethanol) were added and incubated for 2 hours at 25°C. The nitrite produced from the reaction with sulphanilic acid forms a diazonium compound, reacts with α-naphthylamine results a red azo compound was measured at 530 nm and expressed as units (g FW) $^{-1}$.

3.8.3. Analysis of the indices of oxidative damage (MDA content):

Methodology: Heath and Packer, (1968)

	Reagent	Concentration/Volume
Sample preparation	Sample 0.1% TCA (Trichloroacetic acid)	0.5 g 5 ml
Addition of Reagents	20% TCA (containing 0.5% TBA)	Equal aliquot added

Sample was ground in and then centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to a tube, and an equal aliquot was added and heated for 25 minutes at 95°C and then cooled instantly in an ice bath. The solution was cleared by centrifugation for 1 minute. Absorbance was recorded at 532 and 600nm with the nonspecific absorption at 600 nm subtracted from the reading at 532nm. The MDA content was then determined using an extinction coefficient of 155mM $^{-1}$ cm $^{-1}$. Lipid peroxidation was determined from the amount of MDA formed and expressed as mol/g/F.W.

3.8.4 In–vivo visualization of reactive oxygen species:

3.8.4.1 Superoxide radical ($O_2^{\bullet-}$)

The *in–vivo* visualization $O_2^{\bullet-}$ in leaves was examined using the nitro-blue tetrazolium (NBT) dye according to method by Frahry and Schopfer, (2001). Fresh leaves were dipped in 10mM NaN_3 solution, prepared in 100mM buffer containing 0.1% NBT, and incubated for one hour. NBT interacts with superoxide, forming dark blue, insoluble formazan compound. Then, the leaves were washed multiple times with ethanol solution to remove excess stain. Then, the samples were captured in camera.

3.8.4.2 *In-vivo* visualization of H_2O_2:

The visualization H_2O_2 content in each sample was done by the methodology of Thordal-Christensen *et al.,* (1997).

Fresh leaves were thoroughly washed with DW and then dipped in a solution consists of 0.1% DAB acidified HCl to a pH of 3.8. They were incubated for 16h to allow for the uptake of DAB and its reaction with H_2O_2 and peroxidase. The leaves, subsequently bleached by dipping them in ethanol and boiled for 10 min. Photographs were taken, where H_2O_2 was visible in brown coloration.

3.9 Estimation of enzymatic antioxidants:

3.9.1 GST activity:

Methodology: Rakhra *et al.,* (2015)

	Reagent	Concentration/Volume
Reaction Mixture	50 mM Tris-HCl buffer (pH 7.0) 1 mM Ethylene diamine tetra-acetic acid (EDTA) 1 mM CDNB (1-chloro-2,4-dinitrobenzene) 1 mM GSH (Reduced glutathione) Enzyme extract	3 ml (total volume of the reaction) 1 mM 1 mM 1 mM 240 µg

This was measured following the protocol of Rakhra *et al.,* (2015), which involves the GST-catalyzed reaction between reduced glutathione (GSH) and GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB) to form conjugate (2,4-dinitrophenyl) glutathione-S (DNPGS). The change in absorbance was taken at 340 nm and monitored at 5 minutes using a UV- VIS spectrophotometer. One unit of GST activity is the amount of enzyme catalyzing the formation of 1 mmol of DNPGS per minute. Using an extinction coefficient (ε) of 9.6 mM^{-1} cm^{-1}, the specific activity was quantified as millimole of conjugate formed per minute per mg of protein (min^{-1} mg $protein^{-1}$)

$$\text{Specific activity [mmoles min}^{-1}\text{ (mg Protein)}^{-1}\text{]} = \frac{\text{Change in Absorbance min}^{-1} \times \text{Total reaction volume}}{\text{Extinction coefficient} \times \text{Sample volume} \times \text{mg Protein}}$$

3.9.2 GR activity

Methodology: Thomas, (2002)

	Reagent	Concentration/Volume
Reaction mixture	Tris-HCl buffer (pH 7.0) GSSG (Oxidized glutathione) NADPH $MgCl_2$ Enzyme extract	1 ml 1 mM 0.15 mM 3 mM 240 µg

This activity was determined by coupling the oxidation to the lowering of oxidized glutathione (GSSG) with the measurement of this reduction conducted at 340 nm, as

described by Thomas, (2002). The absorbance was taken for 5 minutes at 340 nm. One unit of enzyme activity is estimated as mmol of $NADP^{+-1}$. This activity was quantified as the millimole of $NADP^{+}$ formed or NADPH oxidized min^{-1} mg $protein^{-1}$ (ε= 6.2 mM^{-1} cm^{-1}).

$$\text{Specific activity } [\text{mmoles min}^{-1}\ (\text{mg Protein})^{-1}] = \frac{\text{Change in Absorbance min}^{-1} \times \text{Total reaction volume}}{\text{Extinction coefficient} \times \text{Sample volume} \times \text{mg Protein}}$$

3.9.3 APX activity

Methodology: Barka, (2001)

	Reagent	**Concentration/Volume**
Reaction Mixture	Tris-HCl buffer (pH 7.0)	1 ml
	Ascorbic acid (ascorbate)	0.2 mM
	H_2O_2	20 µM
	EDTA	0.2 mM
	Enzyme extract	240 µg

This activity was determined by the formation of monodehydroascorbate, through the hydrogen peroxide dependent oxidation of ascorbate. The change in optical density (OD) was monitored at 290 nm for 5 minutes using a spectrophotometer. 1 unit is described as the millimole of ascorbate oxidized/min, and specific activity is described as the mmol of ascorbate oxidized per minute per mg of protein (min^{-1} mg $protein^{-1}$) with extinction coefficient (ε) of 2.8 mM^{-1} cm^{-1}.

$$\text{Specific activity } [\text{mmoles min}^{-1}\ (\text{mg Protein})^{-1}] = \frac{\text{Change in Absorbance min}^{-1} \times \text{Total reaction volume}}{\text{Extinction coefficient} \times \text{Sample volume} \times \text{mg Protein}}$$

3.9.4 CAT activity

Methodology: Barka, (2001)

	Reagent	Concentration/Volume
Reaction Mixture	10 mM Hydrogen peroxide (H_2O_2)	1 ml
	50 mM Tris-HCl buffer (pH 7.0)	1 ml
	Enzyme extract	240 µg

CAT activity was estimated using the method described the conversion of H_2O_2 into water and oxygen. This was performed by monitoring the lowering of hydrogen peroxide at 240 nm over 5 minutes using a double beam UV-VIS spectrophotometer. CAT activity (1 unit) was described as the enzyme's amount used to catalyze the oxidation of 1mmol of H_2O_2/min. This activity was quantified as mmol of hydrogen peroxide which was oxidized per mg of protein (min^{-1} mg $protein^{-1}$), with an extinction coefficient (ε) of 39.4 mM^{-1} cm^{-1}.

$$\text{Specific activity } [\text{mmoles min}^{-1} (\text{mg Protein})^{-1}] = \frac{\text{Change in Absorbance min}^{-1} \times \text{Total reaction volume}}{\text{Extinction coefficient} \times \text{Sample volume} \times \text{mg Protein}}$$

3.9.5 GPX activity

Methodology: Rakhra *et al.,* (2015)

Step	Reagent	Concentration/Volume
Reaction Mixture	Tris-HCl buffer (pH 7.0)	1 ml
	Guaiacol	10 mM
	H_2O_2	5 mM
	Enzyme extract	240 µg

GPX activity was employing guaiacol as a substrate and measuring absorbance at 470 nm. The GPX activity was observed at 470 nm by tracking the formation of the tetraguaiacol for 5 minutes using a double beam UV-VIS spectrophotometer. One unit of enzyme activity is the mmol of tetraguaiacol produced per minute. This was quantified in terms of specific activity which was quantified as the millimole of tetraguaiacol formed or hydrogen peroxide reduced min^{-1} mg $protein^{-1}$ (ε= 26.6 mM^{-1} cm^{-1}).

$$\text{Specific activity [mmoles min}^{-1}\text{ (mg Protein)}^{-1}\text{]} = \frac{\text{Change in Absorbance min}^{-1} \times \text{Total reaction volume}}{\text{Extinction coefficient} \times \text{Sample volume} \times \text{mg Protein}}$$

3.9.6 SOD activity

Methodology: Thomas, (2002)

	Reagent	Concentration
Reaction Mixture	Tris-HCl buffer (pH 7.0)	50 mM
	EDTA	3 µM
	Methionine	14.5 mM
	Nitro blue tetrazolium (NBT)	2.25 mM
	Riboflavin	60 µM
	Enzyme extract	240 µg

The whole reaction begins by exposing the tubes for 15 minutes under fluorescent lamps. Then, kept the tubes in the dark for some time (10 minutes), this reaction in the tubes was halted, and the absorbance was measured at 560 nm using a double-beam UV-VIS spectrophotometer.

Enzyme activity

Reduction = (Absorbance of sample/ Absorbance of control) * 100 = y

Inhibition (X) = 100-y

50% inhibition = 1 unit

X% inhibition = 1/50*X = Z units

Specific activity [Units (mg protein)$^{-1}$ = Z units (mg protein)$^{-1}$

4.0 Statistical analysis:

This study utilized data comprising the means and standard errors from three replicates (n = 3) to assess result accuracy. ANOVA was performed using SPSS-16 software to determine the significance between the control and treatment group means. DMRT was utilised at a significance level of $p \leq 0.05$. Before conducting the DMRT analysis, a multivariate test was applied to confirm the variance homogeneity conditions.

CHAPTER 4

RESULTS

4.1 Effect on morphology and photosynthetic pigments:

The present study investigated the impact of salinity and the role of different signaling molecules (H_2S and NO) on plant growth in cucumber seedlings. All the parameters (FW, PH, and DW) of growth diminished significantly under salt stress exposure in the cucumber seedlings. Specifically, FW, PH and DW decreased by 24%, 22%, 26% respectively, under salt stress (**Figure 4.1-4.4)**. However, exogenous application of NaHS resulted an increase of FW, PH, and DW by 14.75%, 7%, 12% while SNP resulted an increase of these parameters by 17.70%, 10%, 18% respectively compared to NaCl treatment. The addition of NaHS and SNP in combination further improved the response compared to individual treatments, depicting an overall improvement of growth parameters by 22% 12%, 23% respectively as compared to NaCl treatment.

The treatments involving L-NAME and cPTIO consistently negated the protective effects of NaHS and SNP, highlighting the critical role of NO in mitigating NaCl-induced stress. Specifically, in presence of L-NAME, the protective effects of NaHS and SNP were significantly reduced, with FW, PH, and DW decreasing by 15%, 7% and 13% in NaCl+ NaHS+ L-NAME treated seedlings as compared to NaCl+ NaHS treated seedlings and 18%, 10%, 19% in NaCl+ NaHS+ L-NAME as compared to NaCl+SNP treated seedlings respectively. Similarly, cPTIO partially inhibited the effects of NaHS and SNP, reducing the FW, PH, and DW by 6.5%, 9.4%, 7.4% in NaCl+ NaHS+ cPTIO and by 13.5%, 14%, 21.5% in NaCl+ SNP+ cPTIO respectively.

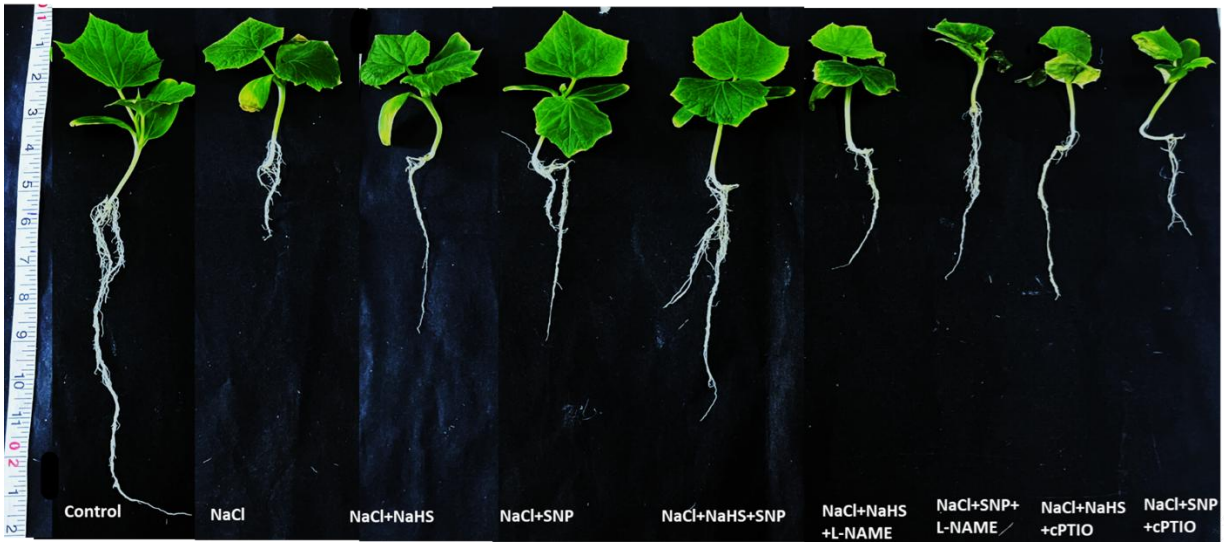


Figure 4.1: Visual effects of different treatment combinations on the growth parameters of cucumber seedlings.

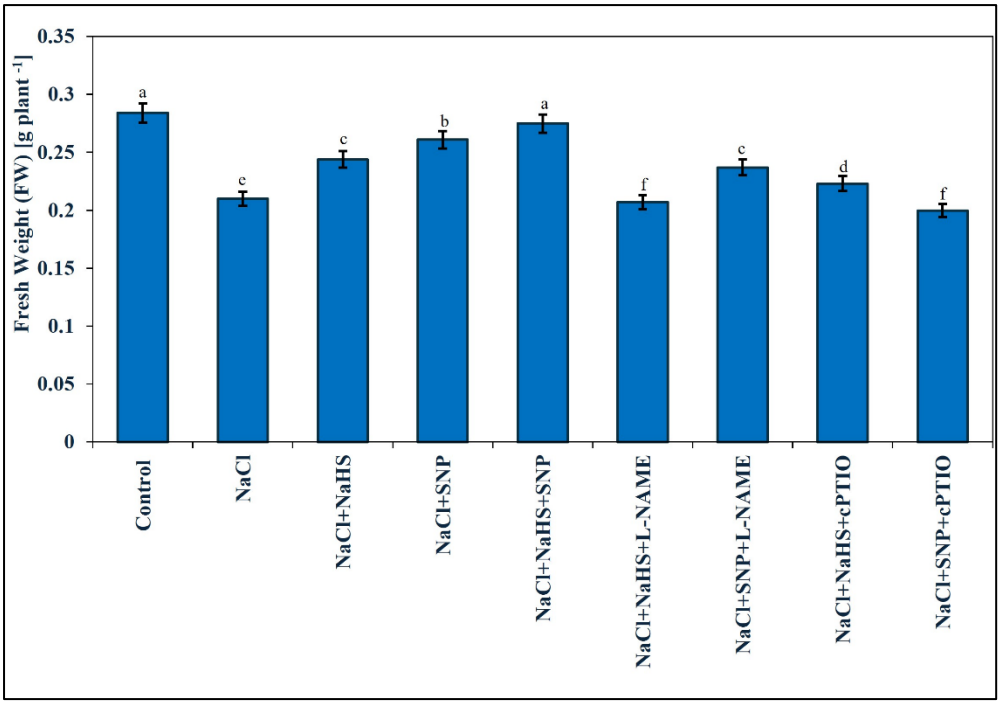


Figure 4.2: Effect of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) in salt-stressed cucumber seedlings on fresh weight (FW). Results are provided as Means ± Standard error of three replicates (n = 3). DMRT (Duncan Multiple Range Test) study of one-way ANOVA reveals significant differences across letters ($p < 0.05$).

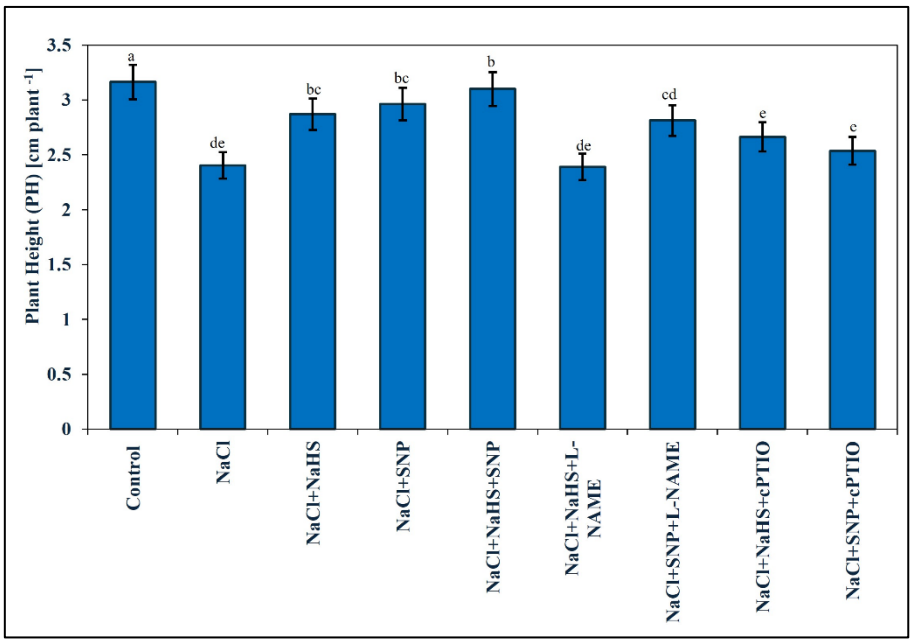


Figure 4.3: Effect of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) in salt-stressed cucumber on plant height (PH). Results are provided as Means ± Standard error of three replicates (n = 3). DMRT (Duncan Multiple Range Test) study of one-way ANOVA reveals significant differences across letters ($p < 0.05$).

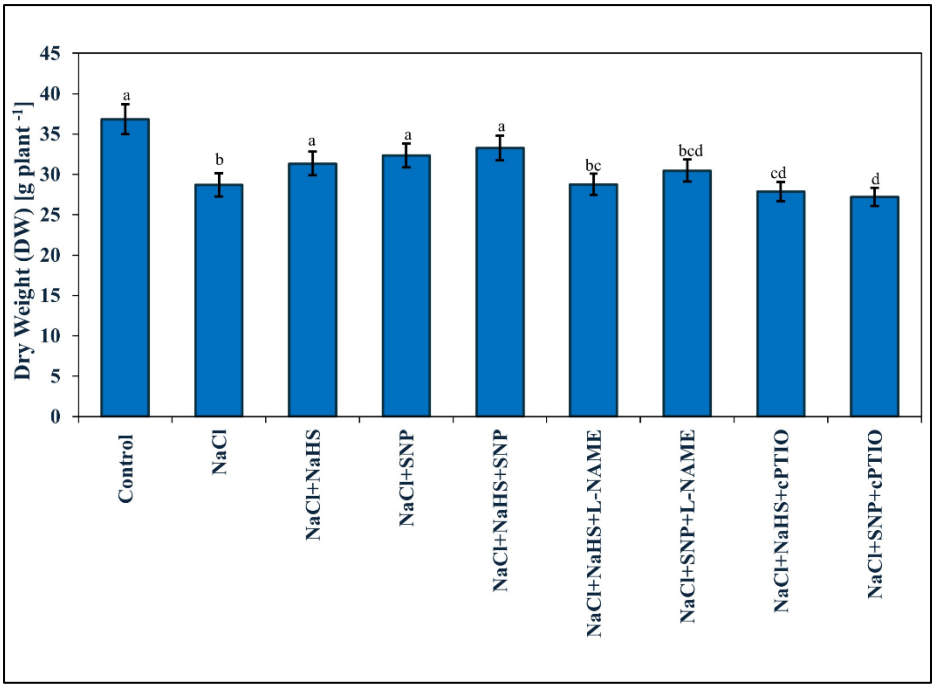


Figure 4.4: Effect of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) in salt-stressed cucumber seedlings on dry weight (DW). Results are provided as Means ± Standard error of three replicates (n = 3). DMRT (Duncan Multiple Range Test) study of one-way ANOVA reveals significant differences across letters ($p < 0.05$).

In bitter gourd seedlings, FW, PH, and DW decreased by 19%, 19%, and 22%, respectively under salt stress. However, exogenous application of NaHS alleviated these effects, increasing FW, PH, and DW by 8.5%, 4.3%, and 18%, respectively, compared to NaCl-treated plants. Similarly, SNP treatment resulted in increases by 10.6%, 7%, and 23% in these parameters, respectively. Notably, the combined application of NaHS and SNP further enhanced growth, leading to improvements by 16%, 12%, and 27% in FW, PH, and DW, respectively, compared to NaCl alone **(Figure 4.5-4.8).**

However, in NaCl + NaHS + L-NAME-treated seedlings, FW, PH, and DW reduced by 11%, 12%, and 10%, respectively, compared to NaCl + NaHS-treated seedlings. Similarly, in NaCl + SNP + L-NAME treated seedlings, reductions by 13%, 15%, and 15% respectively in FW, PH, and DW, were observed. The inhibitory effects of cPTIO were also evident, partially diminishing the improvements achieved by NaHS and SNP. The growth parameters (FW, PH, and DW) reduced by 9%, 14.4%, and 31%, respectively, in NaCl + NaHS + cPTIO treatments

as compared to NaCl+ NaHS treated seedlings, and by 16%, 21.5%, and 45% in NaCl + SNP + cPTIO treatments in comparison to NaCl+ SNP treated cucumber seedlings.

Figure 4.5: Visual effects of different treatment combinations on the growth parameters of Bitter gourd seedlings

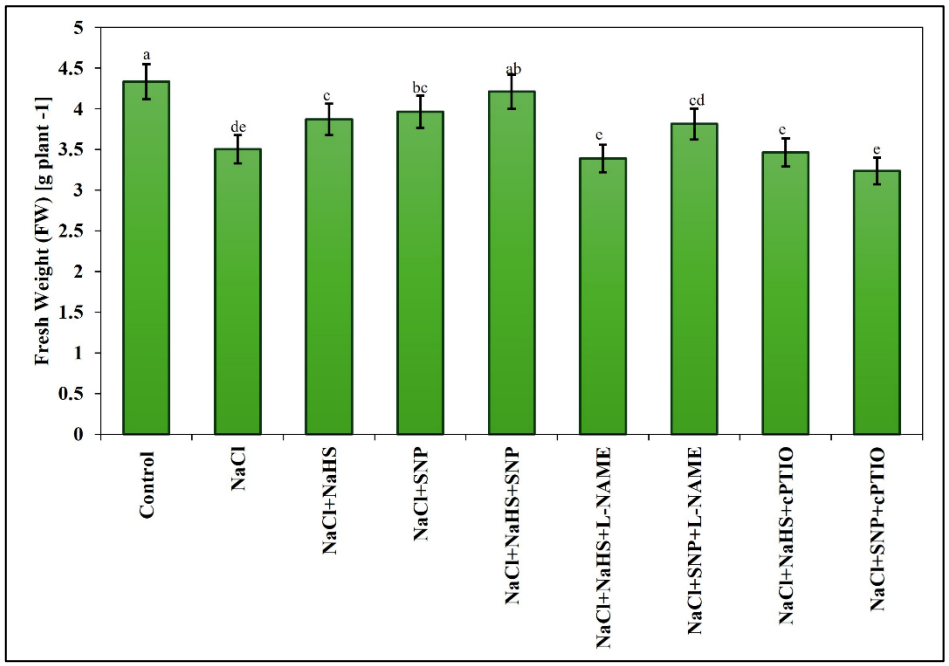


Figure 4.6: Effect of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) in salt-stressed bitter gourd seedlings on fresh weight (FW). Results are provided as Means ± Standard error of three replicates (n = 3). DMRT (Duncan Multiple Range Test) study of one-way ANOVA reveals significant differences across letters ($p < 0.05$).

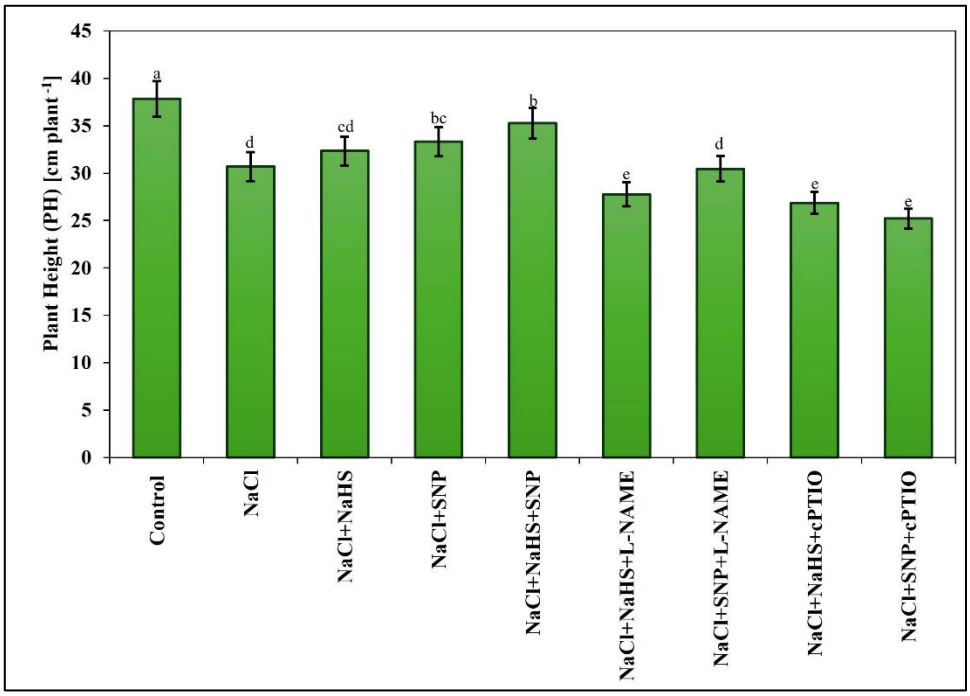


Figure 4.7: Effect of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) in salt-stressed bitter gourd seedlings on plant height (PH). Results are provided as Means ± Standard error of three replicates (n = 3). DMRT (Duncan Multiple Range Test) study of one-way ANOVA reveals significant differences across letters ($p < 0.05$).

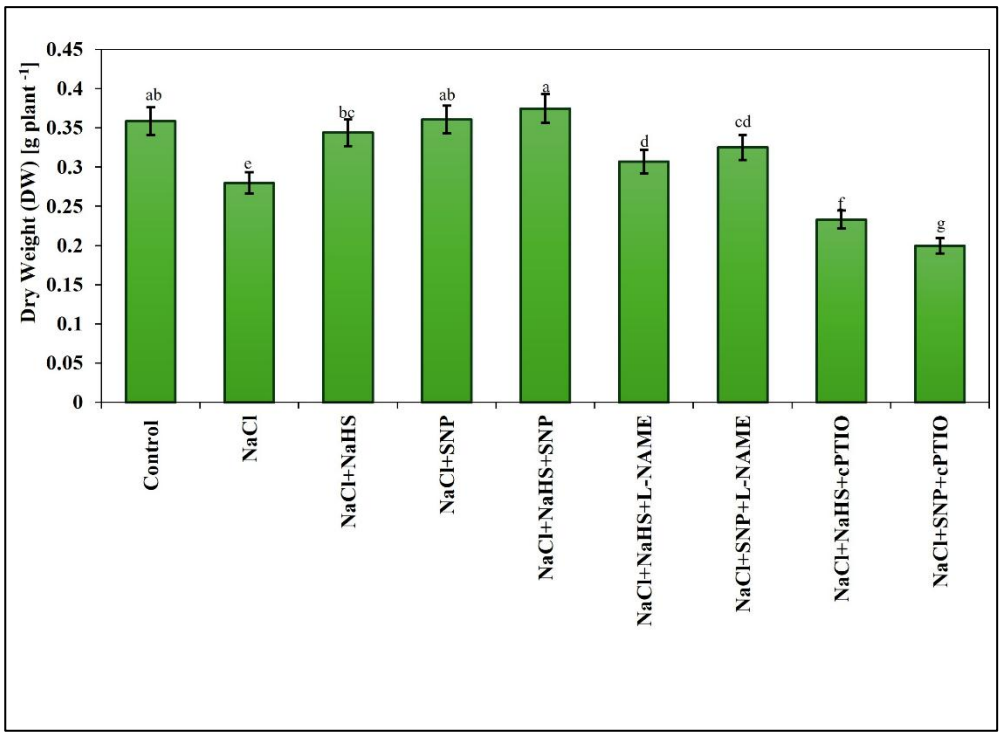


Figure 4.8: Effect of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) in salt-stressed bitter gourd seedlings on dry weight (DW). Results are provided as Means ± Standard error of three replicates (n = 3). DMRT (Duncan Multiple Range Test) study of one-way ANOVA reveals significant differences across letters ($p < 0.05$).

4.2 H_2S and NO repair chlorophyll and carotenoid damage from NaCl toxicity

To understand the signalling role of H_2S and NO under salt stress, different photosynthetic pigments were also analyzed, and the results are summarized in **Figure 4.9**. Under the application of 25 mM NaCl, there was a reduction in Chl a by 18.5%, Chl b by14%, and carotenoids by 22% in cucumber seedlings compared to control one. The role of H_2S and NO in mitigating NaCl-induced stress was examined by treating the seedlings with H_2S and NO donors. There was an increase by 10.8%, 8.2%, and 0.96% in *Chl a, Chl b* and *carotenoids* under NaCl + NaHS treatment setup whereas under NaCl+SNP, there was an increase by 13%, 9.6%, and 7.8% in Chl a, Chl b and car respectively as compared to stress. When treated with the combination of NaCl+NaHS+SNP, the increase in *Chl a, Chl b* and carotenoids by 15%, 13%, and 11% respectively as compared to stressed seedlings. To ascertain the possible role of both these signalling molecules on the photosynthetic machinery, test seedlings were exposed to L-NAME and cPTIO, leading to a noticeable inhibition in pigment contents by 6.2%, 6.8% and 8.1% under NaCl+ NaHS+ L-NAME treatment compared to NaCl + NaHS treated seedlings and by 8.4%, 8.2% and 14.4% under NaCl +SNP+L-NAME treatment compared to NaCl+ SNP treated seedlings. Similarly, seedlings exposed by NaCl + NaHS+ cPTIO led to the decline in the pigment content by 5.3%, 9% and 9.6% and by 11%, 14% and 20% under NaCl+SNP+cPTIO treatment as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

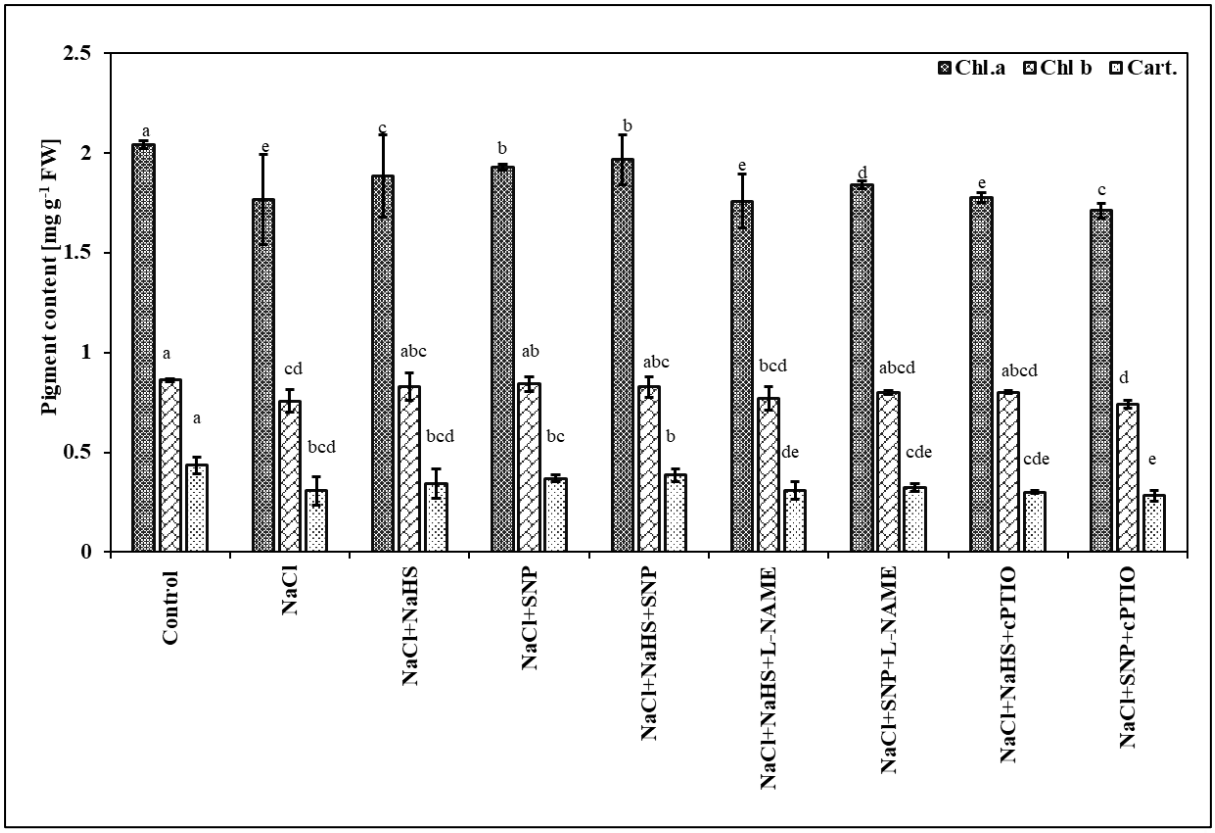


Figure 4.9: Effect of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) on salt-stressed cucumber seedlings' chlorophyll a, b, and carotenoids. Results are provided as Means ± Standard error of three replicates (n = 3). DMRT (Duncan Multiple Range Test) study of one-way ANOVA reveals significant differences across letters ($p < 0.05$).

The bitter gourd seedlings subjected to salt stress also had significant damage to their light-harvest pigments and this was evident in the decrease of chlorophyll a, chlorophyll b and carotenoid contents by 16%, 15.4%, and 31.4% respectively as compared to control values (**Figure 4.10)**. In the bitter gourd seedlings, chlorophyll pigments (Chl a, Chl b and Carotenoids) enhanced by 1.9%, 8.24%, 13% respectively under NaCl + NaHS and by 9.3%, 9.4%, 18% respectively under NaCl + SNP compared to the stressed seedlings. Moreover, NaHS and SNP administered in combination further enhanced the pigments by 11.8%, 10.6% and 14.4% than individual treatments. To investigate the potential role of signalling molecules on the photosynthetic machinery, test seedlings were exposed to L-NAME and cPTIO, which resulted in inhibition of pigment contents by 5.11%, 6.7% and 22% under NaCl+ NaHS+ L-NAME treatment compared to NaCl + NaHS treated seedlings and by 12.6%, 7.9% and 22.7% under NaCl+ SNP+L-NAME treatment compared to NaCl + NaHS treated seedlings. Similarly, when seedlings were exposed to NaCl+NaHS+cPTIO, their pigment content decreased by

2.69%, 5% and 22.5%, and by 13%, 11%, and 32.7% under NaCl+SNP+cPTIO treatment as compared to NaCl+ NaHS+ cPTIO and NaCl+ SNP+ cPTIO treated seedlings.

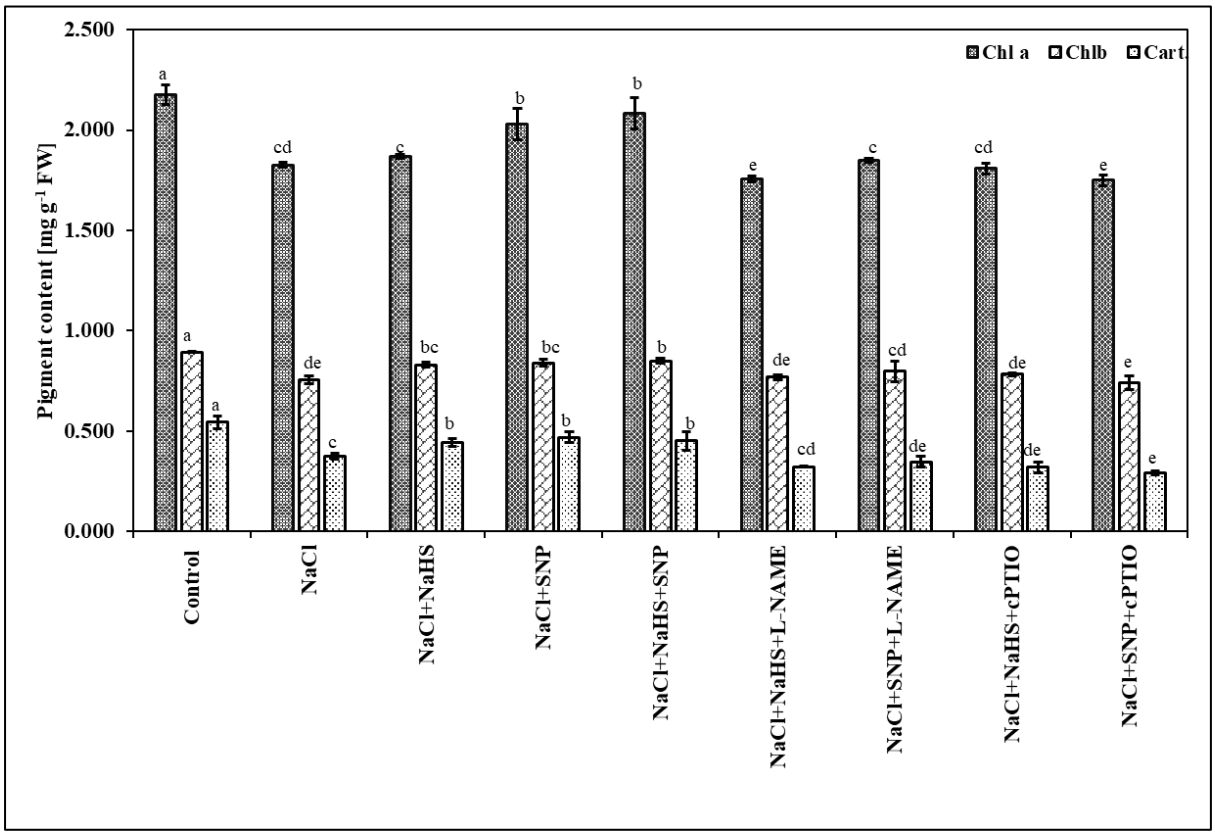
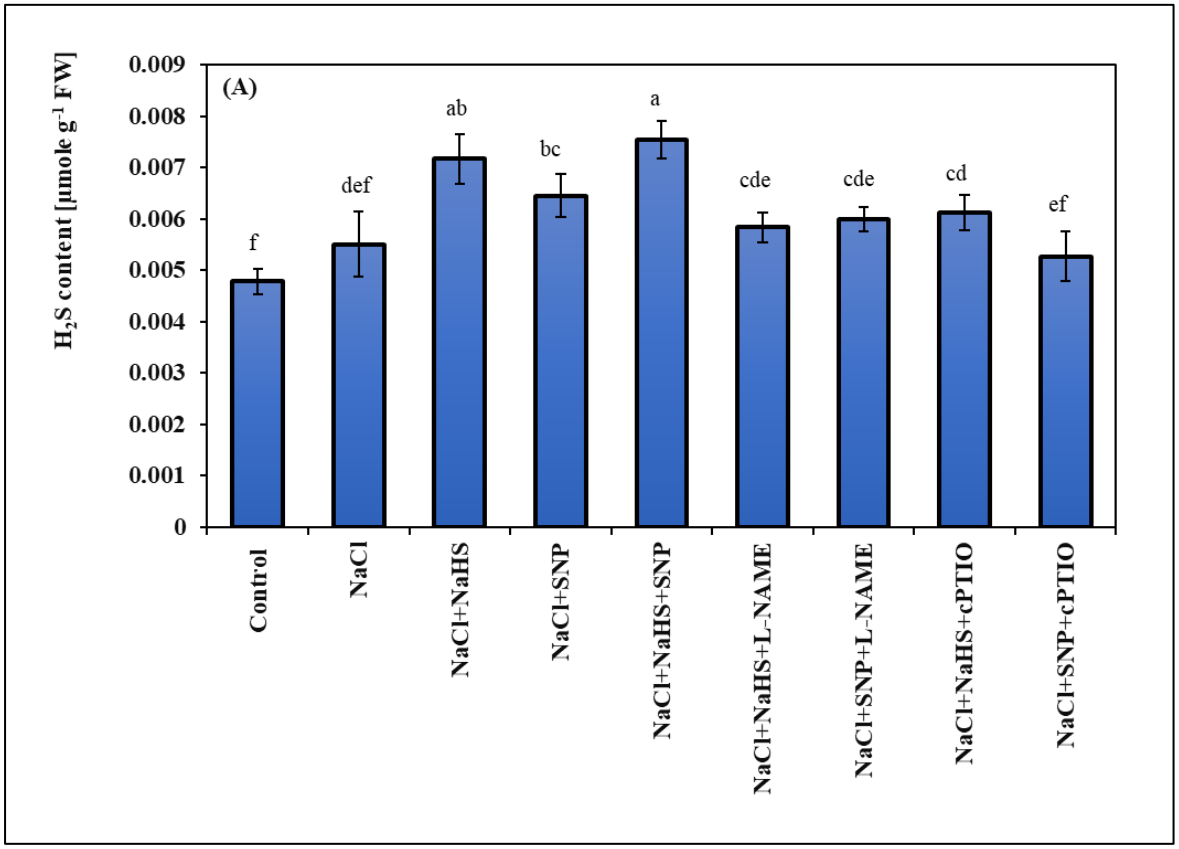


Figure 4.10: Effect of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) on salt-stressed bitter gourd seedlings' chlorophyll a, b, and carotenoids. Results are provided as Means ± Standard error of three replicates (n = 3). DMRT (Duncan Multiple Range Test) study of one-way ANOVA reveals significant differences across letters ($p < 0.05$).

4.3 Integration of H_2S and NO increases resistance under salt stress in cucumber seedlings

To evaluate the effect of NaCl toxicity on the levels of H_2S and NO signalling molecules, endogenous H_2S and NO contents were measured in the leaves of cucumber seedlings grown under the salt stress. The cucumber seedlings resulted a significant increase in the levels of endogenous H_2S and NO by 15% and 48% respectively, as compared to the control plants under saline conditions **(Figure 4.11)**. Besides, the addition of exogenous NaHS to NaCl treated seedlings, led to a notable enhancement in the levels of H_2S and NO by 34.7% and 58% whereas NaCl+ SNP led to an increase H_2S and NO levels by 24% and 64% in salt-stressed seedlings compared to NaCl treated seedlings. In addition, the combination of NaCl+ NaHS+ SNP resulted in further enhancements of endogenous H_2S and NO by 42.5% and 95% in the salt-stressed seedlings compared to individual treatments. In our study, H_2S and NO


(A)
H2S content [µmole g-1 FW]
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
f
def
ab
bc
a
cde
cde
cd
ef
Control
NaCl
NaCl+NaHS
NaCl+SNP
NaCl+NaHS+SNP
NaCl+NaHS+L-NAME
NaCl+SNP+L-NAME
NaCl+NaHS+cPTIO
NaCl+SNP+cPTIO

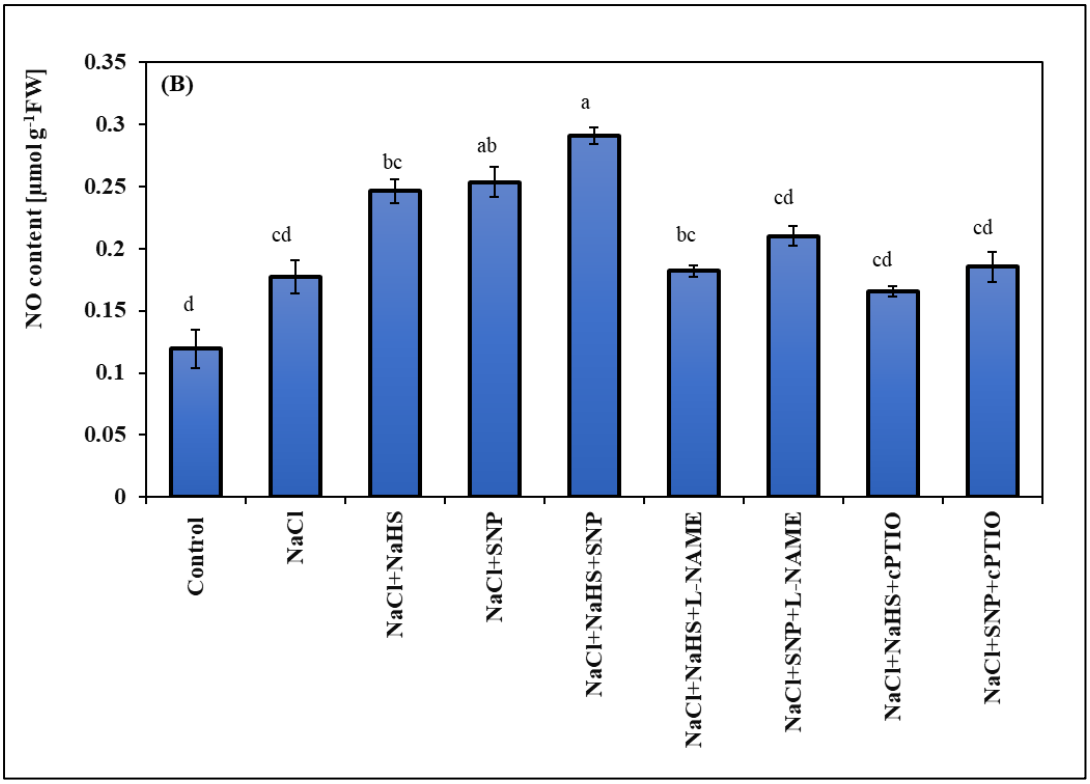


Figure 4.11: The impact of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) through a nutrient solution on H_2S (A) and NO (B) in salt-stressed cucumber seedlings. The results presented are the means ± standard error of three replicates (n=3). The DMRT (Duncan Multiple Range Test) analysis of the one-way ANOVA indicates that the letters exhibit a significant difference with a p-value of less than 0.05.

content was observed to deteriorate by 28% and 54% respectively under the application of NaCl+ NaHS + L-NAME compared to NaCl+ NaHS and by 17% and 60% under the application of NaCl+ SNP+ L-NAME in comparison to NaCl+ SNP stressed seedlings. Remarkably, the H_2S and NO led to a decline by 22% and 68% by the treatment of NaCl+ NaHS + cPTIO and by 29%, 57% in NaCl+ SNP + cPTIO respectively, as compared to NaCl+ NaHS and NaCl+ SNP stressed cucumber seedlings respectively.

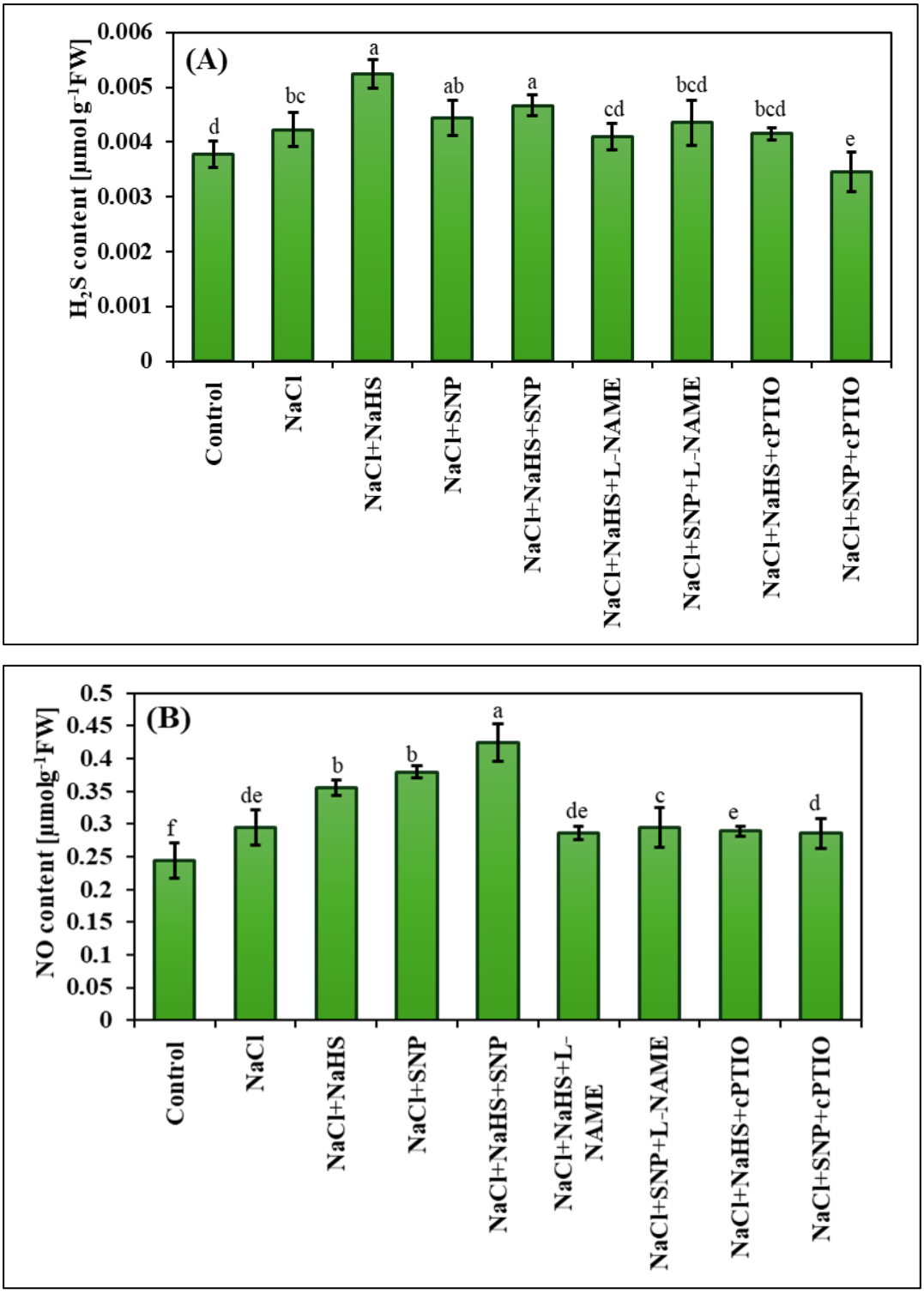


Figure 4.12: The impact of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) through a nutrient solution on H_2S (A) and NO (B) in salt-stressed bitter gourd seedlings. The results presented are the means ± standard error of three replicates (n=3). The DMRT (Duncan Multiple Range Test) analysis of the one-way ANOVA indicates that the letters exhibit a significant difference with a p-value of less than 0.05.

The bitter gourd seedlings exhibited a substantial increase in endogenous H_2S and NO levels by 10.5% and 21%, respectively, under the salt-stress conditions compared to the control plants **(Figure 4.12)**. Besides, addition of exogenous NaHS led to a notable enhancement in the levels of H_2S and NO by 12%, 25% while addition of exogenous SNP led to an increase of H_2S and NO by 3.2%, 35% in salt-stressed seedlings. In addition, the combination of NaHS and SNP resulted in the further enhancement of endogenous H_2S and NO by 10% and 53% respectively, in the salt-stressed seedlings. In our study, H_2S and NO content was observed to be inhibited by 18 % and 29 % respectively under the application of NaCl+ NaHS + L-NAME compared to NaCl+ NaHS whereas NaCl+ SNP+ L-NAME resulted in decrease by 10% and 38% as compared to NaCl+ SNP stressed seedlings. Remarkably, the H_2S and NO declined by 14% and 27.4% by the treatment of NaCl+ NaHS + cPTIO and by 28%, 39% in NaCl+ SNP + cPTIO respectively, in salt-stressed bitter gourd seedlings.

4.4 Impact of nitrogen metabolism:

4.4.1 Inorganic nitrogen content:

(a) Nitrate (NO_3^-) content:

Figure 4.13 shows the effect of NaCl treatment on NO_3^- content in cucumber seedlings, showing a decrease by 20% in cucumber seedlings. However, seedlings when exposed to H_2S and NO through NaHS and SNP individually, led to a notable enhancement in the nitrate content by 6% and 10% respectively. But when NaHS and SNP were applied in combination, the NO_3^- content was furthermore increased by 12% than NaCl treatment seedlings. Additionally, treatment of seedlings with L-NAME as NAHS+ L-NAME and SNP+ L-NAME reduced NO_3^- content by 9% and 13% respectively under salt stress. Using cPTIO (NaHS+ cPTIO and SNP+ cPTIO) to block the signaling led to the reduction in NO_3^- by 8% and 17% respectively, under salinity as compared to the signalling molecules NaCl+NaHS and NaCl+ SNP respectively.

Figure 4.14 presents the impact of NaCl treatment on NO_3^- content in bitter gourd seedlings, showing a 20% decrease as compared to control. However, this reduction was mitigated when the stressed seedlings were treated either with NaHS and/or SNP, resulting in an increased nitrate content by 5% and 7%, respectively. When NaHS and SNP were applied together with NaCl, the NO_3^- content was further increased by 11% compared to NaCl treatment. Nevertheless, treatment with L-NAME (NaCl+ NaHS + L-NAME and NaCl+ SNP + L-NAME) reduced the NO_3^- content by 10% and 11%, respectively, as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings. The use of cPTIO (NaCl+ NaHS + cPTIO and NaCl+ SNP

+ cPTIO) resulted by 7% and 12% decrease in NO_3^- content, respectively as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings under saline conditions.

(b) Nitrite (NO_2^-) content:

The NaCl exposure significantly decreased the NO_2^- content in the leaves of test seedlings of cucumber by 17% over the value of control. On the other hand, the application of NaHS and SNP significantly ameliorated the negative effect caused by the stress, thereby upregulating the nitrite content by 6.5% and 9% respectively. In the combined form (NaCl+ NaHS+ SNP), NO_2^- content was further upregulated by 14% as compared to salt stressed seedlings (**Figure 4.13)**. To ascertain the effect of NO, NOS and NR specific inhibitors were used and a sharp reduction by 12 % and 15% in the NO_2^- content was recorded in L-NAME treated samples (NaCl+ NaHS+ L-NAME and NaCl+ SNP+ L-NAME) respectively as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings under saline conditions. However, in the setups treated with cPTIO, a sharp decline in NO_2^- content was noticed (9% in NaCl+ NaHS+ cPTIO and 12% in NaCl+ SNP+ cPTIO) as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings under saline conditions.

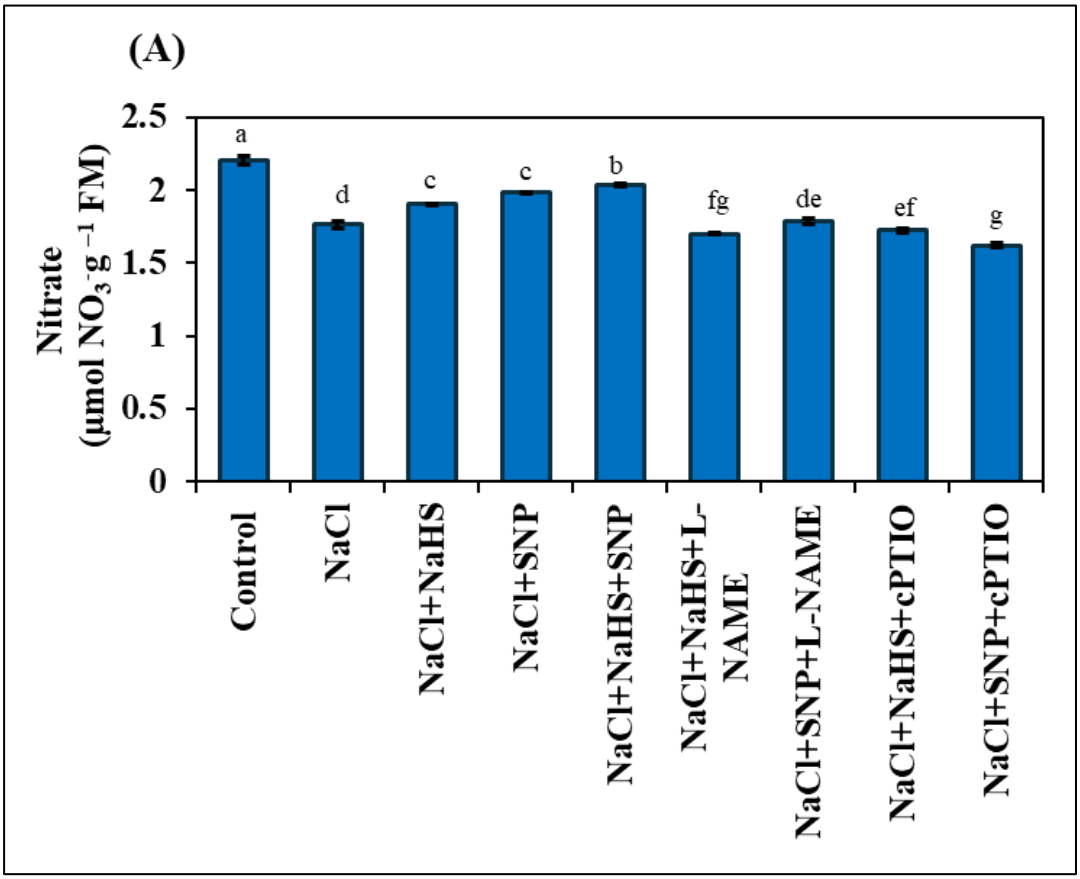
NaCl exposure significantly reduced NO_2^- content in bitter gourd seedling leaves by 19%. However, treatment with NaCl+ NaHS and NaCl+ SNP effectively alleviated the negative effect by increasing NO_2^- content by 9% and 10%, respectively. Furthermore, when NaCl+ NaHS + SNP were applied together, NO_2^- - content was further increased by 15% as compared to the stress conditions **(Figure 4.14)**. On the contrary, in the L-NAME-treated samples, NO_2^- content was reduced by 13% in NaCl+ NaHS + L-NAME and by 14% in NaCl+ SNP + L-NAME as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings. Similarly, a marked decline in NO_2^- content was observed in the cPTIO-treated seedlings, with reduction by 11% in NaCl+ NaHS + cPTIO and 15% in NaCl+ SNP + cPTIO under salinity as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

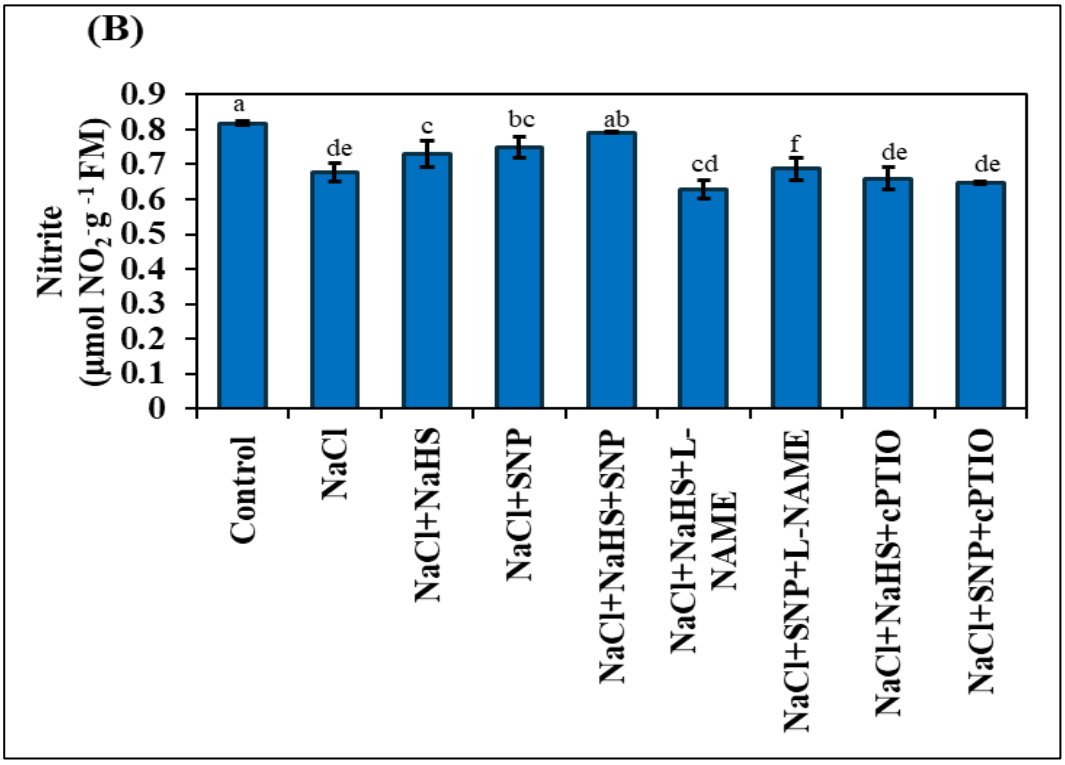
(c) **Ammonium (NH_4^+) content:**

The results related to ammonium (NH_4^+) content is presented in **Figure 4.13.** Unlike NO_3^- and NO_2^- levels, NH_4^+ content increased under NaCl treatment by 38% in cucumber seedlings as compared to the control. Additionally, exogenous NaCl+ NaHS and NaCl+ SNP application significantly reduced the NH_4^+ content by 19% and 23% as compared to stressed seedlings. However, the treatment of NaCl+ NaHS+ SNP, intensified the reduction in ammonia content by 33% in stressed seedlings as compared to stressed seedlings. The seedlings treated with L-

NAME showed a marked increase by 25% in NaCl+ NaHS+ L-NAME and 29% in NaCl+ SNP+ L-NAME as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings. Similarly, the use of cPTIO exacerbated the toxicity, increasing NH_4^+ content by 11% in NaCl+ NaHS+ cPTIO and 21% in NaCl+ SNP +cPTIO in cucumber seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

In comparison to the control, the NH_4^+ content of bitter gourd seedlings increased by 43% under NaCl treatment. Contrarily, the NH_4^+ content was reduced by 20% and 29%, respectively, by the exogenous application of NaHS and SNP to the stressed seedlings. Nevertheless, the ammonia content reduced significantly by 34% as compared to NaCl. under the combined application of NaHS+ SNP treatment **(Figure 4.14)**. In the same vein, seedlings that were subjected to NaCl stress and treated with L-NAME exhibited a substantial increase in NH_4^+ content by 26% in NaCl+ NaHS+ L-NAME and by 34% in NaCl+ SNP+ L-NAME as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings. Likewise, in cPTIO treated seedlings, a marked increase by 9% and 25% respectively in NaCl+ NaHS+ cPTIO and NaCl+ SNP +cPTIO was observed as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.




(B)
Nitrite (µmol NO_2^- g^{-1} FM)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
de
c
bc
ab
cd
f
de
de
Control
NaCl
NaCl+NaHS
NaCl+SNP
NaCl+NaHS+SNP
NaCl+NaHS+L-NAME
NaCl+SNP+L-NAME
NaCl+NaHS+cPTIO
NaCl+SNP+cPTIO

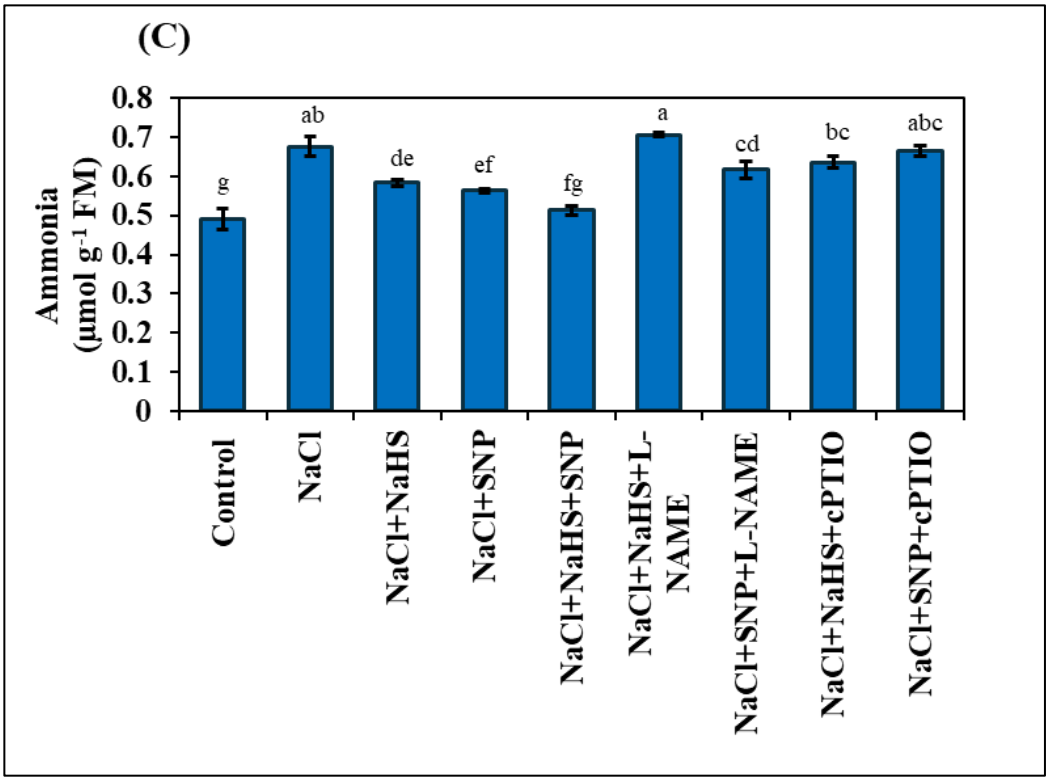
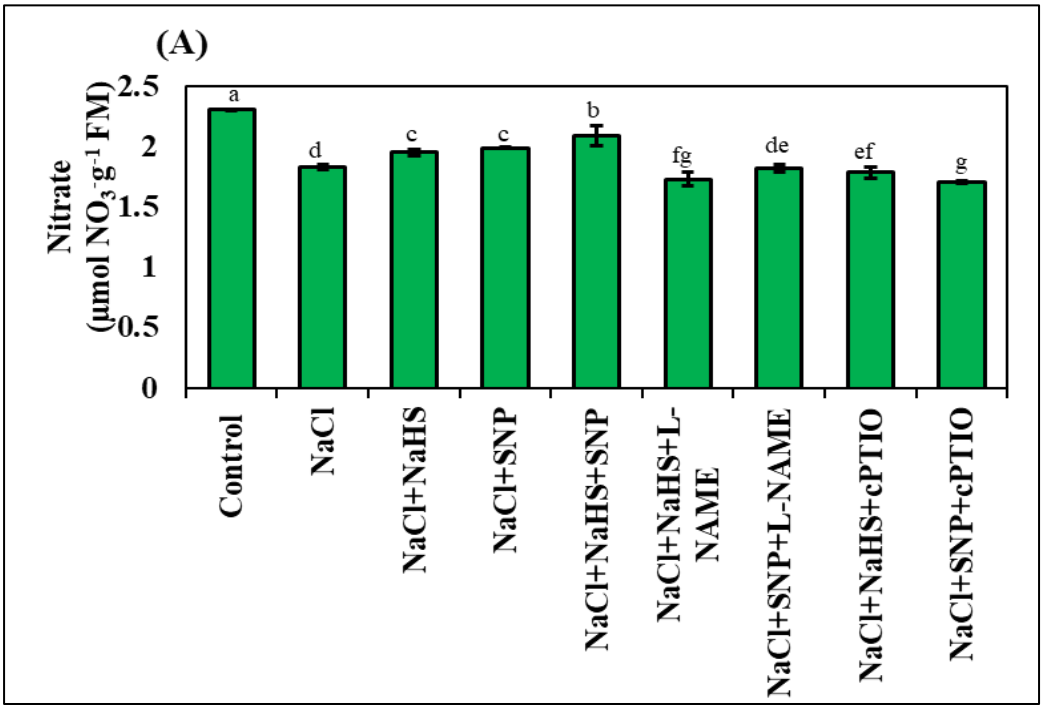
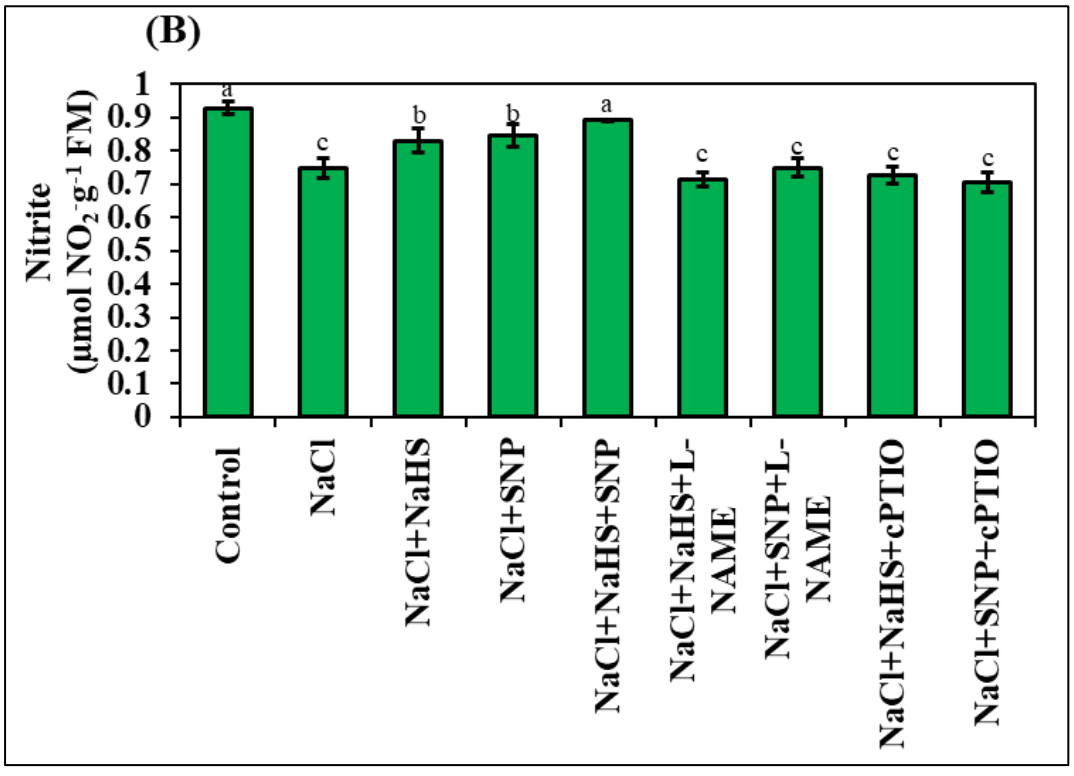

(C)
Ammonia (µmol g^{-1} FM)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
g
ab
de
ef
fg
a
cd
bc
abc
Control
NaCl
NaCl+NaHS
NaCl+SNP
NaCl+NaHS+SNP
NaCl+NaHS+L-NAME
NaCl+SNP+L-NAME
NaCl+NaHS+cPTIO
NaCl+SNP+cPTIO

Figure 4.13: Impact of exogenous supplementation of NaHS alone or together with SNP, L-NAME (inhibitor of NO) and cPTIO, scavenger of NO through the nutrient solution on activities of nitrate (A) and nitrite (B) and ammonia (C) of cucumber seedlings exposed to NaCl stress. The results presented are Means±Standard error of three replicates (n = 3). Different letters show significant $P < 0.05$ difference as per DMRT (Duncan Multiple Range Test) analysis of one-way ANOVA





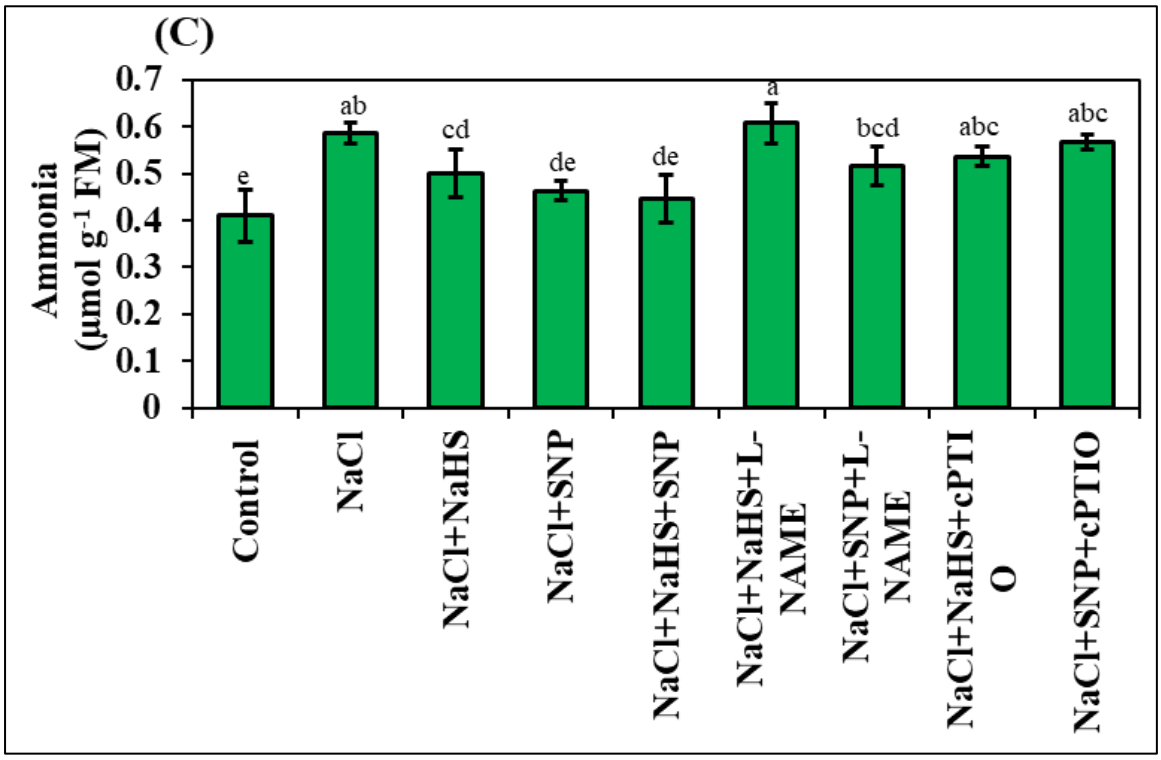
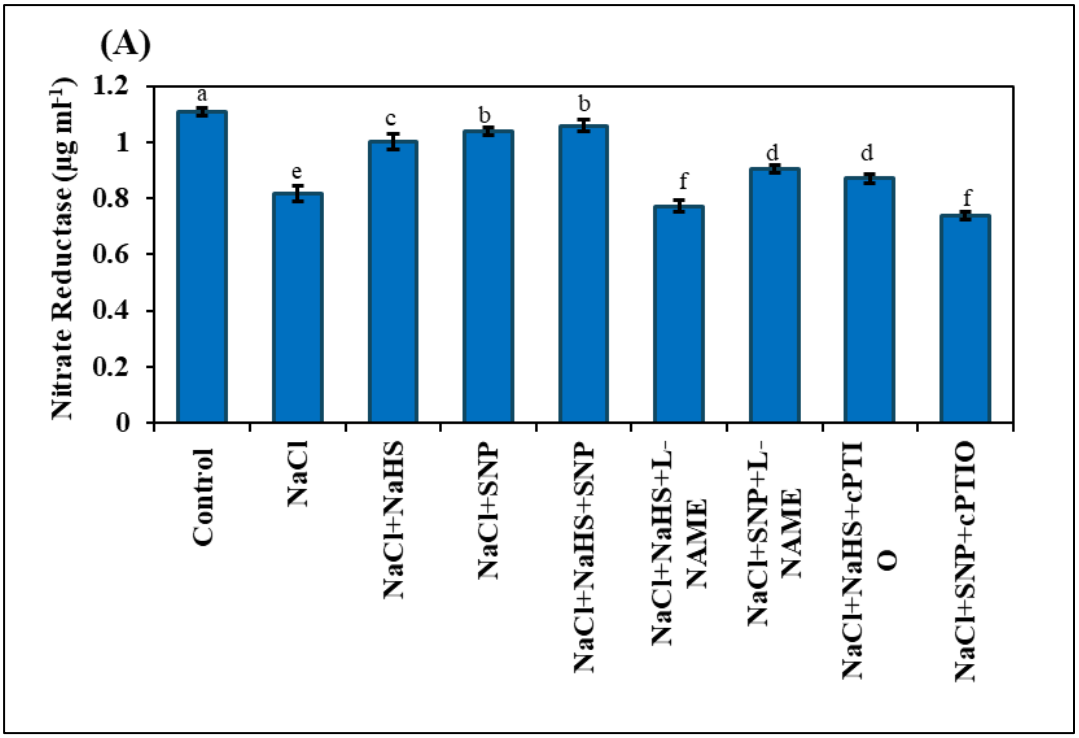


Figure 4.14: Impact of exogenous supplementation of sodium hydrogen sulfide (NaHS) alone or together with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO) and cPTIO, scavenger of NO through the nutrient solution on activities of nitrate (A) and nitrite (B) and ammonia (C) of Bitter gourd seedlings exposed to NaCl stress. The results presented are Means±Standard error of three replicates (n = 3). Different letters show significant $P < 0.05$ difference as per DMRT (Duncan Multiple Range Test) analysis of one-way ANOVA

4.4.2 Nitrate assimilating enzymes:

(a) Nitrate reductase (NR) activity: The results of NR activity are shown in When NaCl was applied, NR activity decreased by 26% in cucumber seedlings compared to the control (**Figure 4.15)**. However, applying NaCl+ NaHS, NaCl+ SNP and NaCl+ NaHS+ SNP either alone or in combination to the seedlings reduced this inhibition, increasing NR activity by 17%, 20% and 20% respectively, as compared to stress in cucumber seedlings.


(A)
Nitrate Reductase (µg ml-1)
1.2
1
0.8
0.6
0.4
0.2
0
a
e
c
b
b
f
d
d
f
Control
NaCl
NaCl+NaHS
NaCl+SNP
NaCl+NaHS+SNP
NaCl+NaHS+L-NAME
NaCl+SNP+L-NAME
NaCl+NaHS+cPTIO
NaCl+SNP+cPTIO

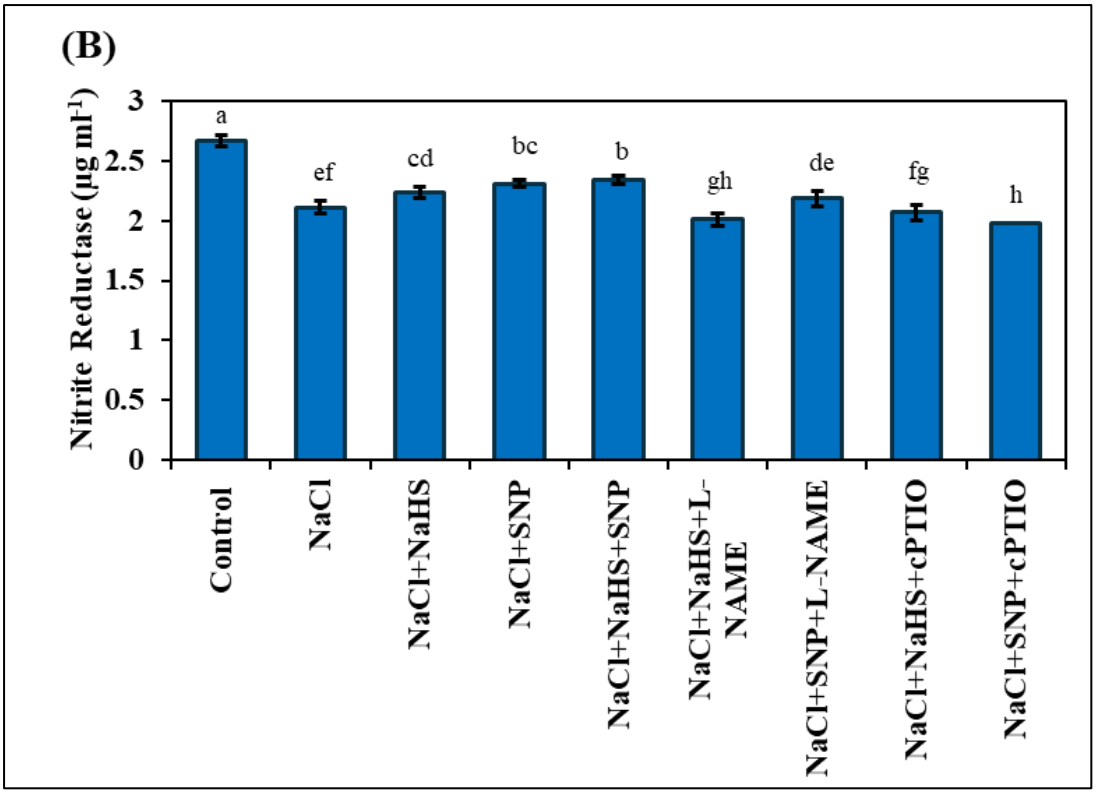


Figure 4.15: Effect of exogenous supplementation of sodium hydrogen sulfide (NaHS) alone or together with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO) and cPTIO, scavenger of NO through the nutrient solution on activities of nitrate reductase (NR) (A) and nitrite reductase (NiR)(B) of cucumber seedlings exposed to NaCl stress. The results presented are Means±Standard error of three replicates (n = 3). Different letters show significant $P < 0.05$ difference as per DMRT (Duncan Multiple Range Test) analysis of one-way ANOVA

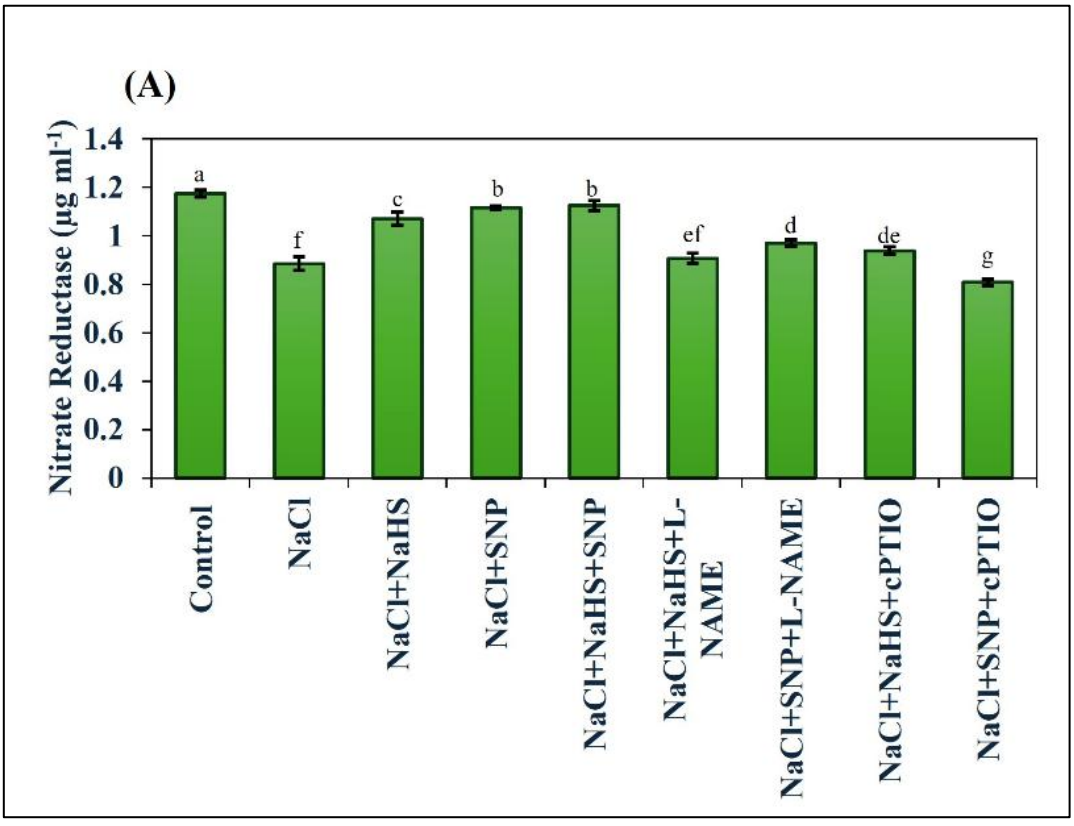
Adding L-NAME along with NaCl worsened the damage, decreasing NR activity by 21% and 24% in NaCl+ NaHS+ L-NAME and NaCl+ SNP+L-NAME, respectively as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings. This effect was stronger in cPTIO-treated seedlings, where NR activity dropped significantly by 12% in NaCl+ NaHS+ cPTIO and 27% in NaCl+ SNP+ cPTIO as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

Figure 4.16 shows the results of nitrate reductase (NR) activity in bitter gourd seedlings. When NaCl was added, the seedlings of bitter gourd showed a 25% decrease in the NR activity as compared with control. The treatment of NaCl+ NaHS, NaCl+ SNP, and their combination removed inhibition and NR activity increased by 16%, 20%, and 21% respectively. However, by 14 % and 18 % inhibition in NR activity was observed in the experimental setup NaCl + NaHS + L-NAME and NaCl + SNP + L-NAME respectively as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings. The inhibitory effect was more significant in the seedlings treated with cPTIO, where NR activity decreased by 11% in NaCl+ NaHS + cPTIO and by 26% in NaCl+ SNP + cPTIO as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

(b) Nitrite reductase (NiR) activity:

The activity of NiR went down by 21% in cucumber seedlings. Though, application of NaHS and SNP individually, showed an increase in activity of NiR by 5% and 7% respectively, and by 9% under NaCl+ NaHS+ SNP treatment in the cucumber seedlings compared to the stress **(Figure 4.15)**. The treatment with cPTIO led to a steep depression in NiR activity, which declined by 6% and 13% respectively in NaCl+ NaHS + cPTIO and NaCl+ SNP + cPTIO under salt stress. Further, the additive treatment of L-NAME with NaCl led to more augmented damage through decreased NiR activity by 9% and 11% respectively, in NaHS + L-NAME and SNP + L-NAME as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

The activity of NiR was reduced by 20% in bitter gourd seedlings **(Figure 4.16)**. However, when NaHS and/or SNP was supplied either alone or in combination, the activity of NiR increased by 4%, 7% and 8% respectively. The treatment of L-NAME with NaCl resulted in enhanced salt damage, as evident from a decrease in the activity of NiR to the extent by 8% in NaCl+ NaHS + L-NAME and 11% in NaCl+ SNP + L-NAME. Likewise, salinity induced a marked decrease in the activity of NiR by 6% in NaCl+ NaHS + cPTIO and 12% in NaCl+ SNP + cPTIO in bitter gourd seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.



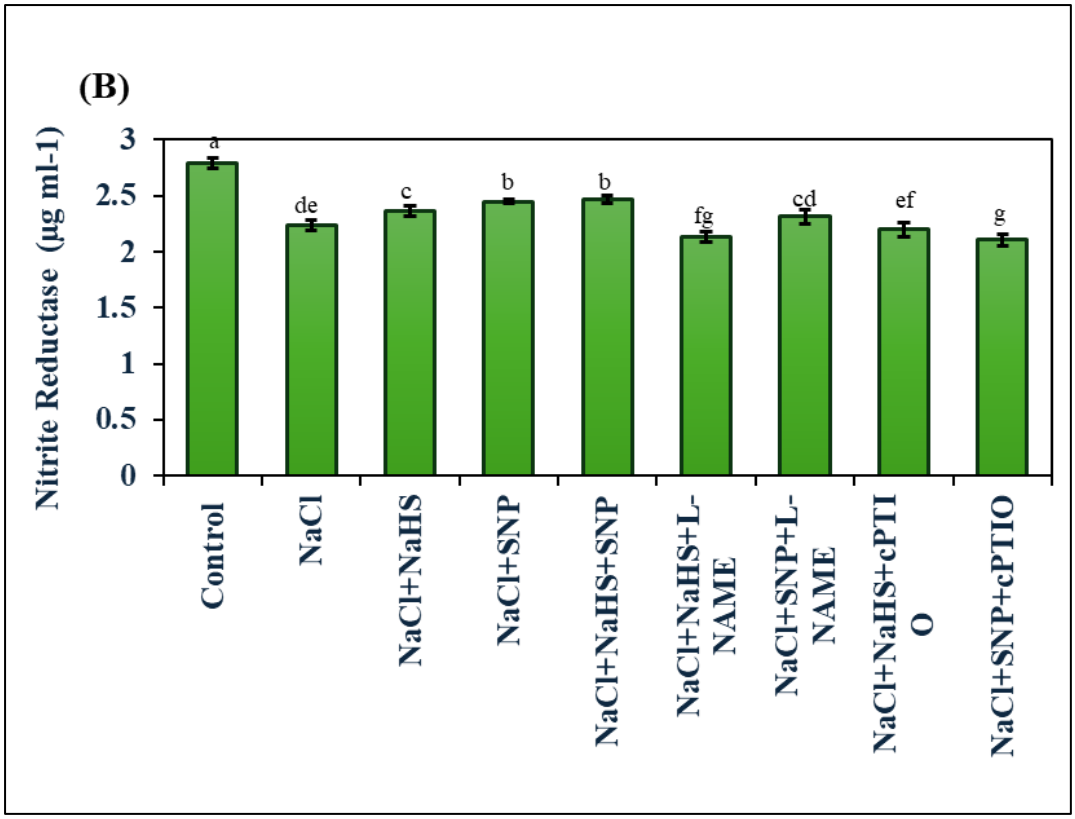


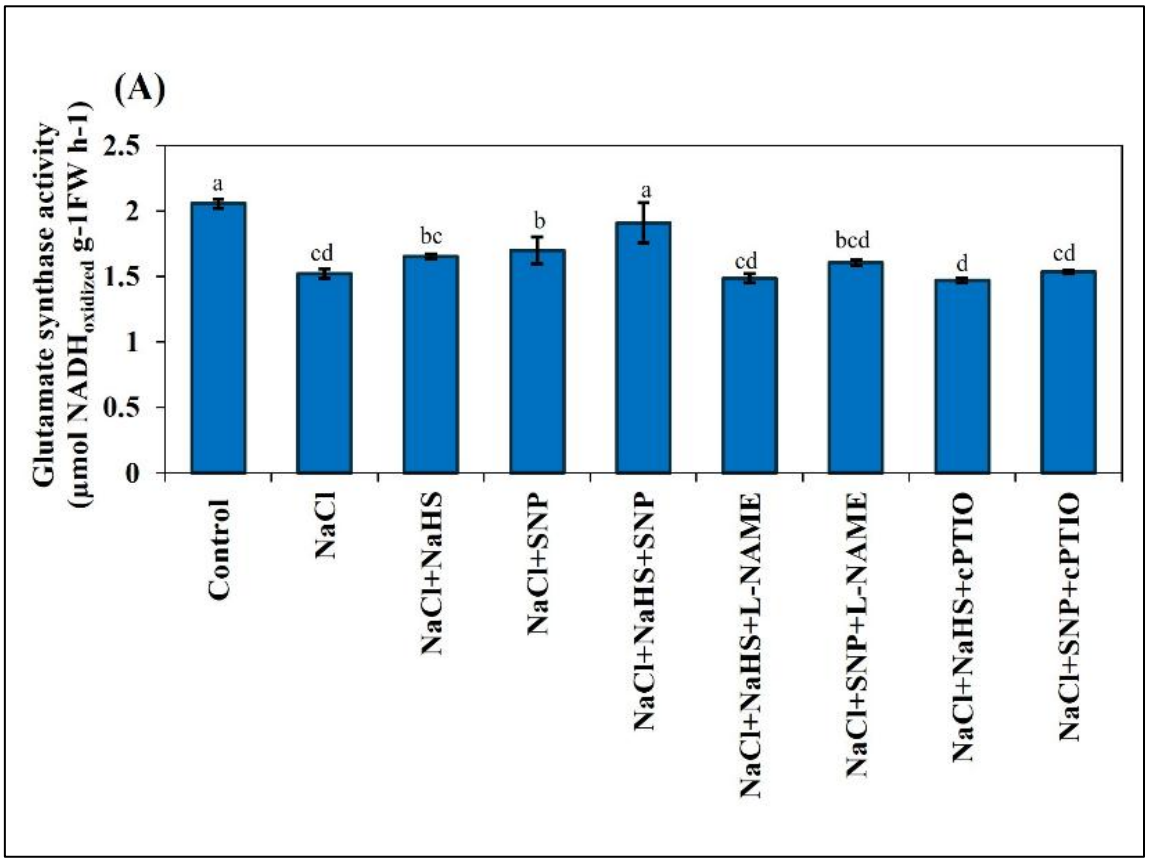
Figure 4.16: Impact of exogenous supplementation of sodium hydrogen sulfide (NaHS) alone or together with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO) and cPTIO, scavenger of NO through the nutrient solution on activities of nitrate reductase (NR) (A) and nitrite reductase (NiR)(B) of bitter gourd seedlings exposed to NaCl stress. The results presented are Means±Standard error of three replicates (n = 3). Different letters show significant $P < 0.05$ difference as per DMRT (Duncan Multiple Range Test) analysis of one-way ANOVA

4.4.3 Ammonium assimilating enzymes:

(a) Glutamate Synthase (GOGAT) activity:

The NaCl treatment lowered the GOGAT activity by 26% in cucumber seedlings compared with the control. Exogenously supplied NaHS and SNP mitigated the adverse effect of stress by upregulating the activity by 6% and 9% in cucumber seedlings as compared to stress conditions. Moreover, the combined treatment of NaCl+ NaHS+ SNP further increased the activity by 19% as compared to stress. But, when NaHS + L-NAME and SNP + L-NAME applied, a decreased GOGAT activity by 8% and 10% respectively observed in salt stressed cucumber seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings. Similarly, treatment with cPTIO also depressed the activity of GOGAT by 9% and 8% in NaHS + cPTIO and SNP + cPTIO respectively as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings (**Figure 4.17**).

In the bitter gourd seedlings also, NaCl reduced GOGAT activity by 27%. The exogenous application of NaHS and SNP significantly alleviated the negative effects by 4% and 10%, respectively as compared to stress. When applied together (NaCl+ NaHS+



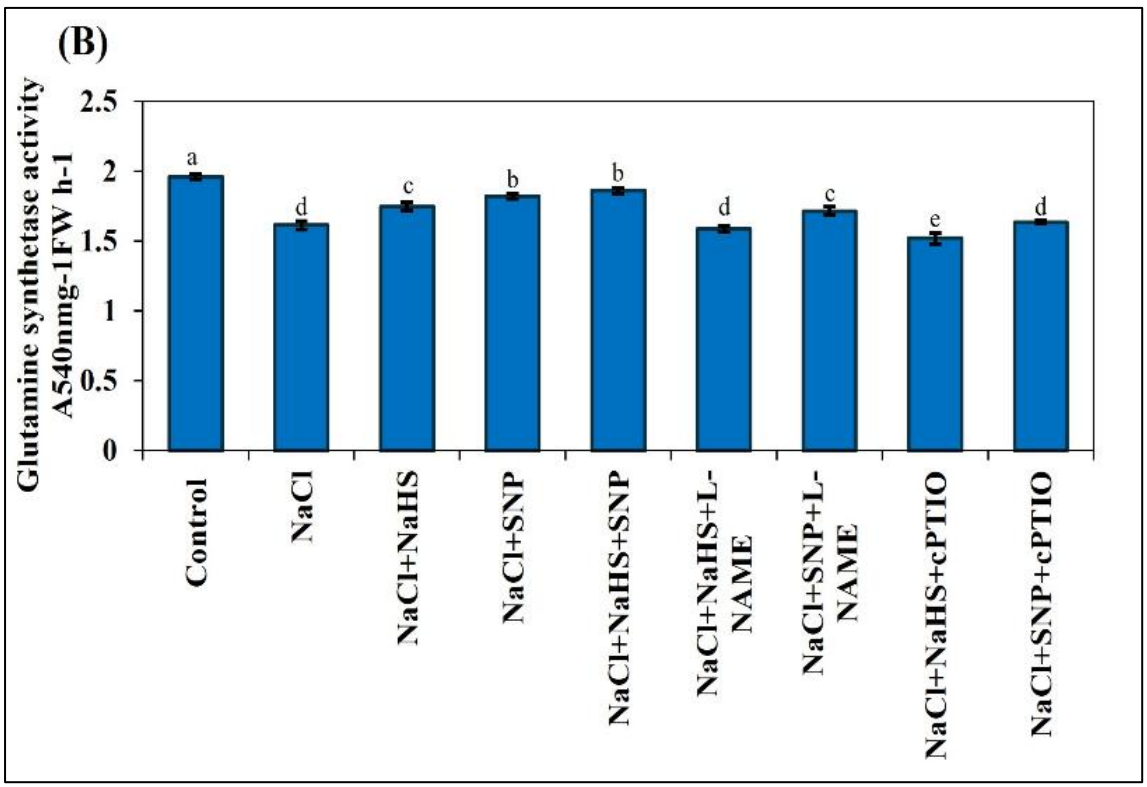


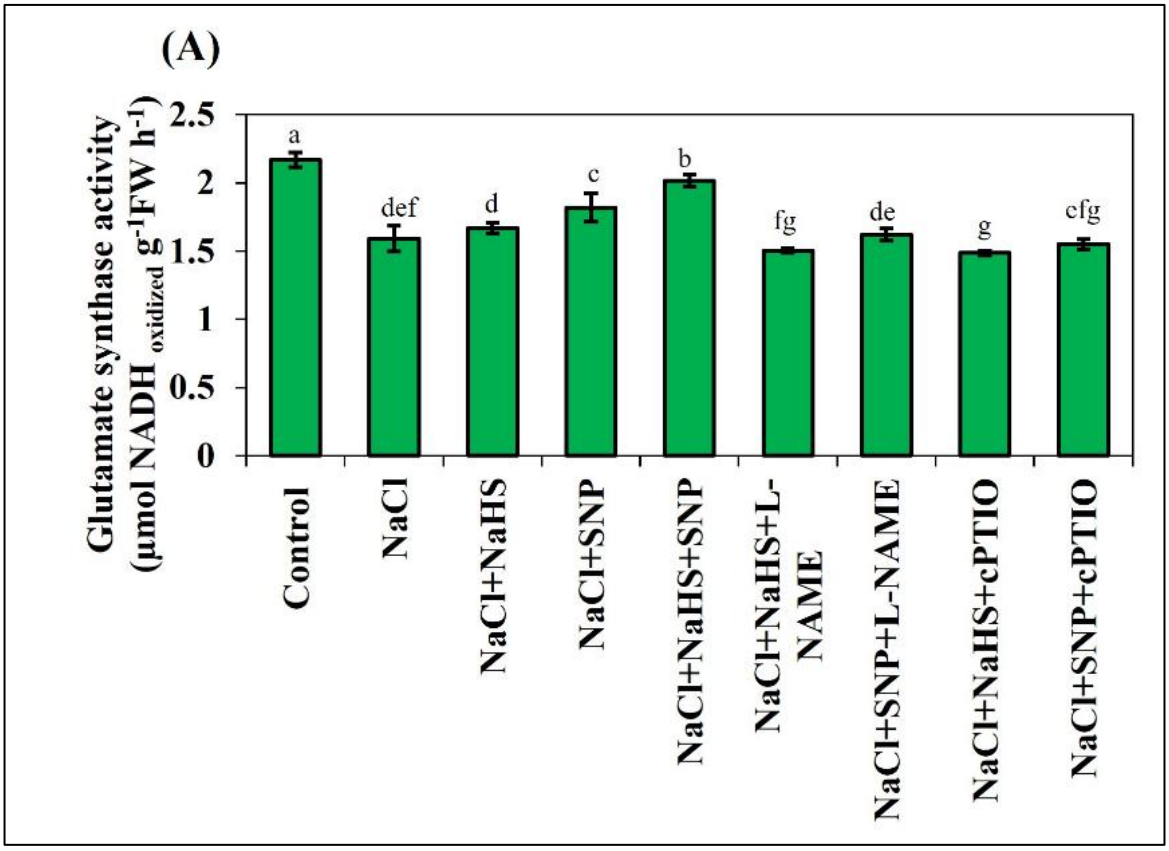
Figure 4.17: Impact of exogenous supplementation of sodium hydrogen sulfide (NaHS) alone or together with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO) and cPTIO, scavenger of NO through the nutrient solution on activities of Glutamate Synthase (GOGAT) (A) and Glutamine Synthetase (GS) (B) of cucumber seedlings exposed to NaCl stress. The results presented are Means±Standard error of three replicates (n = 3). Different letters show significant $P < 0.05$ difference as per DMRT (Duncan Multiple Range Test) analysis of one-way ANOVA

SNP), the treatment further improved GOGAT activity by 20% as compared to stress (**Figure 4.18)**. However, the combined use of L-NAME with NaCl, as seen in NaHS + L-NAME and SNP + L-NAME treatments, led to a more substantial decrease in GOGAT activity by 8% and 15%, respectively. Additionally, treatment with cPTIO further suppressed GOGAT activity, reducing it by 9% in NaHS + cPTIO and by 12% in SNP + cPTIO under as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

(b) Glutamine Synthetase (GS) activity:

In the cucumber seedlings also, NaCl reduced GS activity by 18% compared to the control. The administration of exogenous NaHS and SNP resulted in a substantial increase in the activity of GS by 7% and 11% whereas in combination, this activity further enhanced by 13% as compared to stressed seedlings **(Figure 4.17)**. The treatment with cPTIO depressed the activity of GS by 12% and 10% in NaHS + cPTIO and SNP + cPTIO respectively under stress. Similarly, when NaHS + L-NAME and SNP + L-NAME were combined with NaCl, GS activity decreased by 8% and 12% in given seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

In the presence of NaCl, GS activity reduced by 21% of the control value in bitter gourd seedlings as well. The application of exogenous NaHS and SNP individually resulted in an increase in the activity of GS by 7% and 10% while in combination (NaCl+ NaHS+ SNP), this activity further enhanced by 11% as compared to stress. The treatment with cPTIO depressed the activity of GS by 12% and 9% respectively in NaHS + cPTIO and SNP + cPTIO treated seedlings **(Figure 4.18)**. Likewise, when NaHS + L-NAME and SNP + L-NAME were combined with NaCl, the GS activity decreased by 8% and 11% respectively as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.



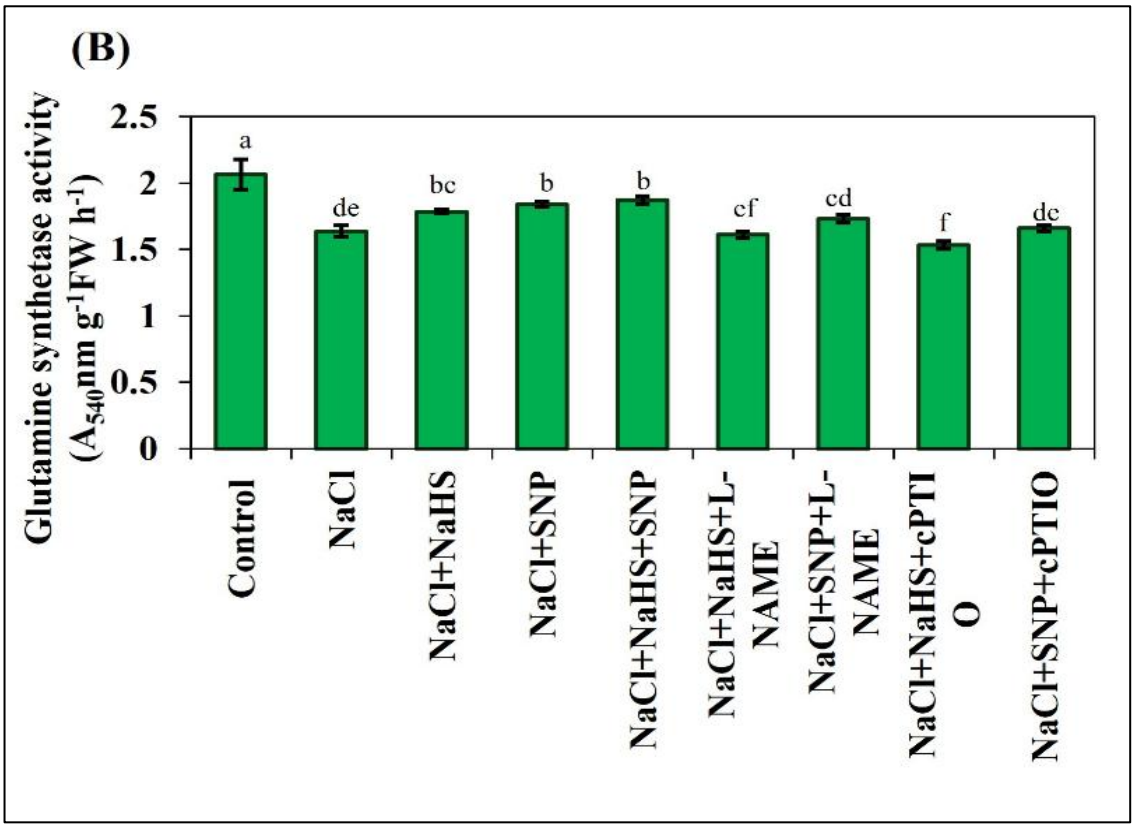


Figure 4.18: Impact of exogenous supplementation of sodium hydrogen sulfide (NaHS) alone or together with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO) and cPTIO, scavenger of NO through the nutrient solution on activities of Glutamate Synthase (GOGAT) (A) and Glutamine Synthetase (GS) (B) of bitter gourd seedlings exposed to NaCl stress. The results presented are Means±Standard error of three replicates (n = 3). Different letters show significant $P < 0.05$ difference as per DMRT (Duncan Multiple Range Test) analysis of one-way ANOVA

4.5 Impact of Oxidative Stress Biomarkers: Reactive Oxygen Species and Oxidative

4.5.1. Reactive oxygen species:

(a) Superoxide radical (SOR; $O_2^{\cdot-}$):

The results depicted in **Figure 4.19** indicate a substantial increase by 73% in the $O_2^{\bullet-}$, under NaCl stress. When the seedlings were supplemented with either NaCl + NaHS or NaCl + SNP, it shows a decline by 25% and 37% in $O_2^{\bullet-}$, as compared to salinity conditions in cucumber seedlings. Moreover, as expected, the treatment of NaCl + NaHS + SNP in combination resulted in a much greater decline by 62% as compared to stressed cucumber seedlings. Nevertheless, in our study, the application of exogenous cPTIO or L-NAME to cucumber seedlings in addition to NaHS and SNP resulted in an elevation of $O_2^{\bullet-}$ by 24 % in NaCl + NaHS + L-NAME, 35% in NaCl + SNP + L-NAME, 2% in NaCl + NaHS + cPTIO and 18% in NaCl + SNP + cPTIO thereby negating the alleviating effect of signalling molecules as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

The data presented in **Figure 4.20** show a significant increase by 52% in $O_2^{\bullet-}$ under NaCl stress compared to control in bitter gourd seedlings. However, when the stressed seedlings were treated with NaHS or SNP, the superoxide radical levels decreased by 20% and 28%, respectively, compared to seedlings exposed to salinity alone. Notably, the combined treatment of NaCl + NaHS + SNP led to a further reduction by 45% in $O_2^{\bullet-}$ levels compared to the stressed seedlings. In contrast, the addition of exogenous cPTIO or L-NAME alongside NaHS and SNP caused a rise in $O_2^{\bullet-}$, with an increase by 25% in NaCl + NaHS + L-NAME, 32% in NaCl +SNP + L-NAME, 5% in NaCl +NaHS + cPTIO, and 9% in NaCl +SNP + cPTIO as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

(b) Hydrogen peroxide (H_2O_2):

The data in **Figure 4.19** shows a 67% increase in H_2O_2 levels in cucumber seedlings under NaCl stress compared to control seedlings. However, treatment with NaCl + NaHS or NaCl + SNP, dropped the H_2O_2 levels by 45% and 59%, respectively, compared to seedlings exposed to salinity. The combined application of NaCl + NaHS + SNP resulted in an additional 83% reduction in H_2O_2 levels compared to the stress conditions. Adding exogenous cPTIO or L-NAME alongside NaHS and SNP increased H_2O_2 levels, with the upregulation by 19% in NaCl + NaHS + L-NAME, 32% in NaCl + SNP + L-NAME, 10% in NaCl +NaHS + cPTIO, and 40% in NaCl +SNP + cPTIO as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

Similarly, in salt-stressed bitter gourd seedlings, NaCl stress caused a 75% increase in H_2O_2 (**Figure 4.20**). Treatment with NaCl + NaHS or NaCl + SNP reduced H_2O_2 levels by 32% and 44%, respectively, compared to the NaCl. The combined NaCl + NaHS + SNP treatment led to a further 62% reduction as compared to stress conditions. However, the addition of cPTIO or L-NAME reversed these effects, increasing H_2O_2 levels by 28% in NaCl+ NaHS + L-NAME, 31% in NaCl+ SNP + L-NAME, 11% in NaCl+ NaHS + cPTIO, and 29% in NaCl+ SNP + cPTIO as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

Furthermore, our study visualized and confirmed the increased levels of $O_2^{\bullet-}$ and H_2O_2 in leaf tissues under salinity stress using nitroblue-tetrazolium (NBT) and DAB stainings, which leads to the blue formazan and brown colour respectively. Under NaCl stress, the intensity of the color was more intense in comparison to control while the application of exogenous H_2S or NO alone or in combination led to a decline in $O_2^{\bullet-}$ content, and, therefore. the intensity of the color decreased noticeably. On the contrary, the application of cPTIO or L-NAME resulted in enhanced levels of $O_2^{\bullet-}$ under salt stress and thereby, the blue formazan and brown color intensified under such conditions **(Figure 4.21,4.22).**

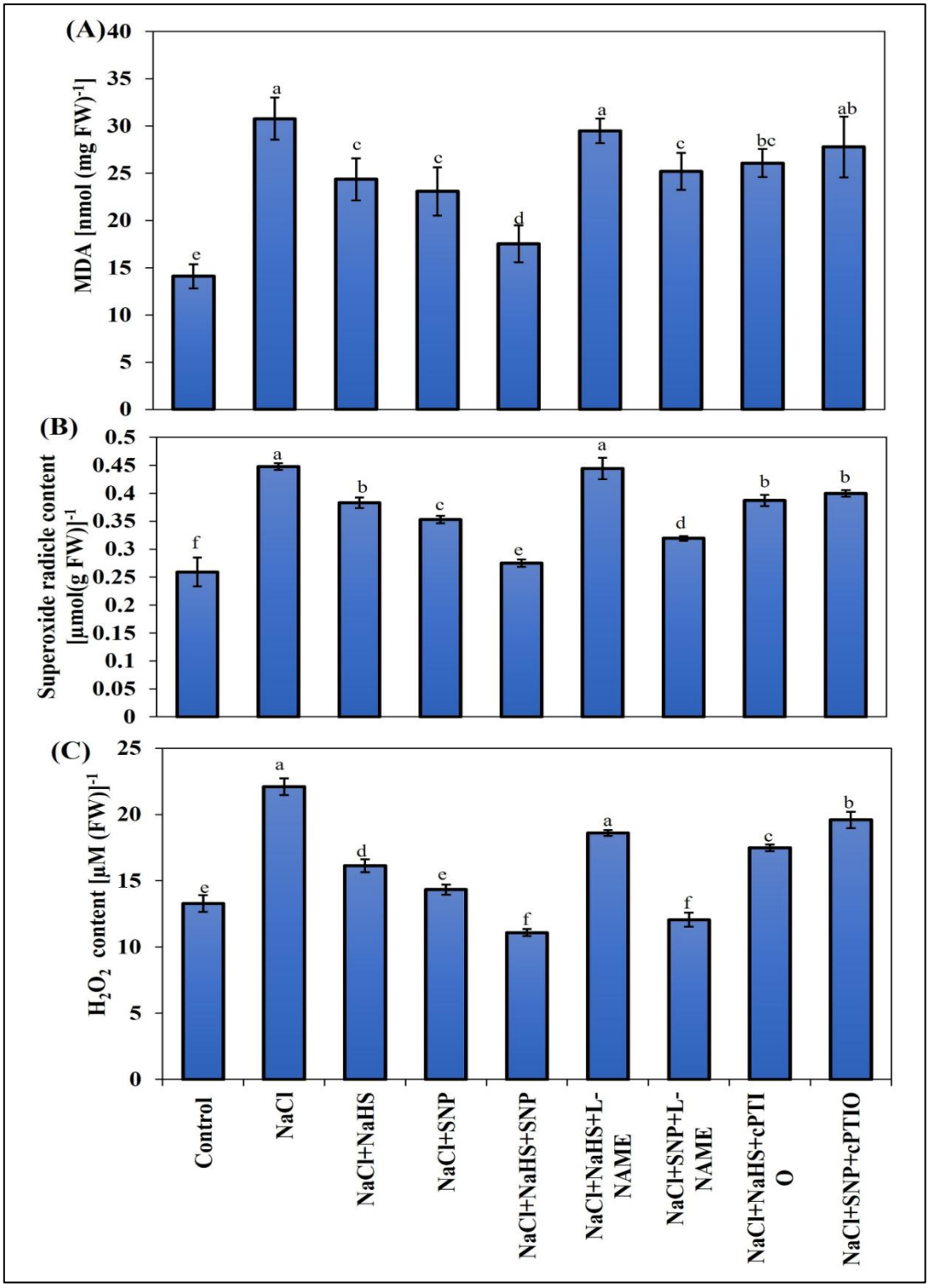


Figure 4.19: The effects of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) through a nutrient solution on MDA (A), superoxide radicle (B) and H_2O_2 (C), in salt-stressed cucumber seedlings. The results presented are the means ± standard error of three replicates (n = 3). The DMRT (Duncan Multiple Range Test) analysis of the one-way ANOVA indicates that the letters exhibit a significant difference with a p-value of less than 0.05.

4.5.2 Indices of oxidative damage:

(a) Lipid peroxidation:

The results related to lipid peroxidation are shown in **Figure 4.19**. NaCl stress led to a 118% increase in MDA content in cucumber seedlings. The application of NaHS and SNP significantly reduced MDA levels by 46% and 55%, respectively, with the combined application of NaHS and SNP resulting in a 94% reduction compared to salinity alone. In contrast, the combination of cPTIO with NaCl increased MDA levels by 12% in NaHS + cPTIO and by 33% in SNP + cPTIO under salt stress. Additionally, treatments with L-NAME also resulted in a significant rise in MDA content by 36% in NaHS+ L-NAME and 45% in SNP+ L-NAME as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings under saline conditions.

NaCl stress led to a 46% increase in MDA content in bitter gourd seedlings. However, the application of NaHS and SNP significantly lowered MDA levels by 33% and 39%, respectively, with a reduction by 66% when NaHS and SNP were applied together as compared to salinity **(Figure 4.20).** Applying cPTIO with NaCl elevated MDA levels by 2% and 22% under NaHS + cPTIO and SNP + cPTIO treatments. Moreover, treatments with L-NAME caused a significant increase in MDA content by 24% in NaHS + L-NAME and 30% in SNP + L-NAME under saline conditions as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

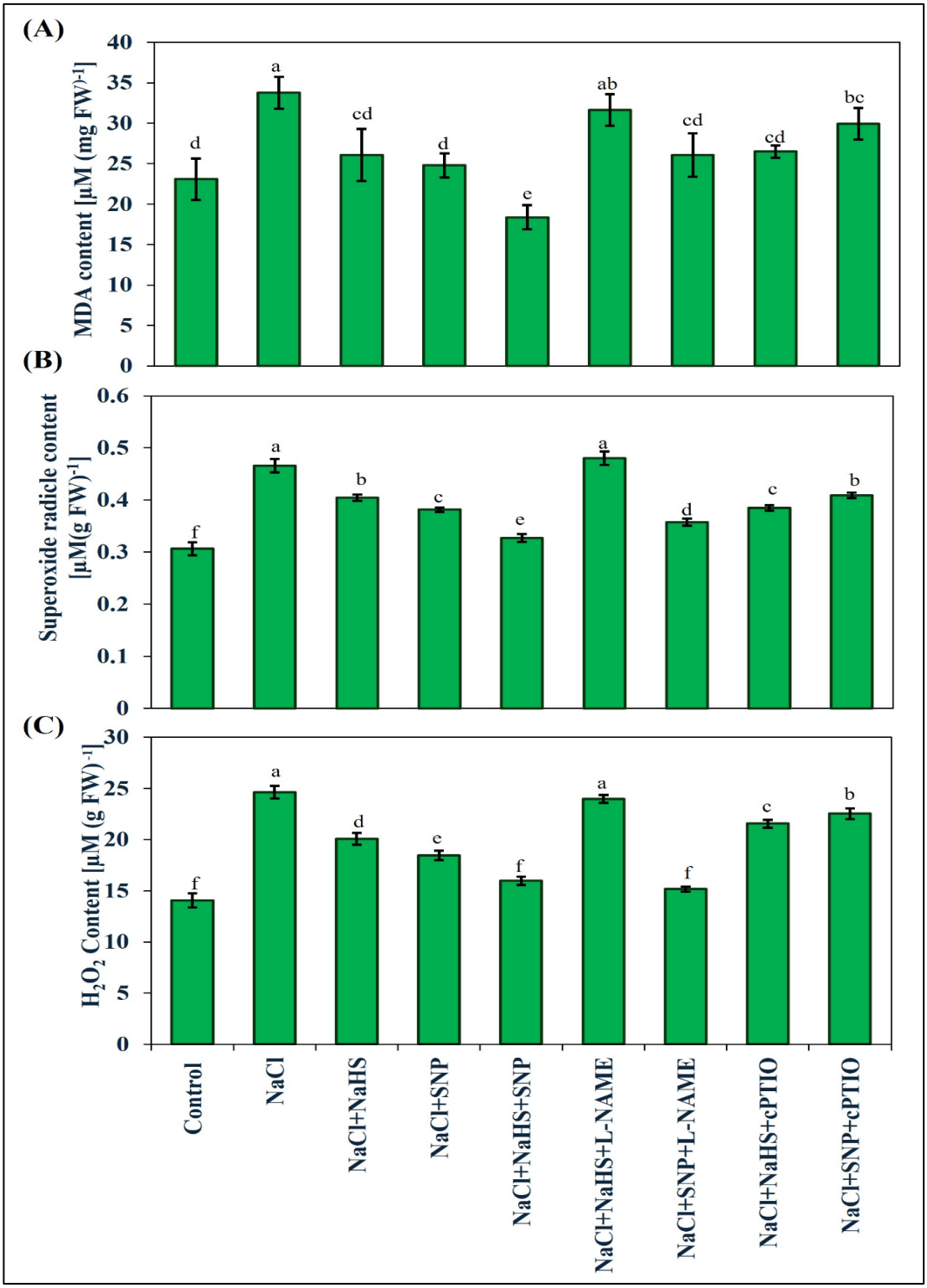


Figure 4.20: The effects of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) through a nutrient solution on MDA (A), superoxide radicle (B) and H_2O_2 (C), in salt-stressed bitter gourd seedlings. The results presented are the means ± standard error of three replicates (n = 3). The DMRT (Duncan Multiple Range Test) analysis of the one-way ANOVA indicates that the letters exhibit a significant difference with a p-value of less than 0.05.

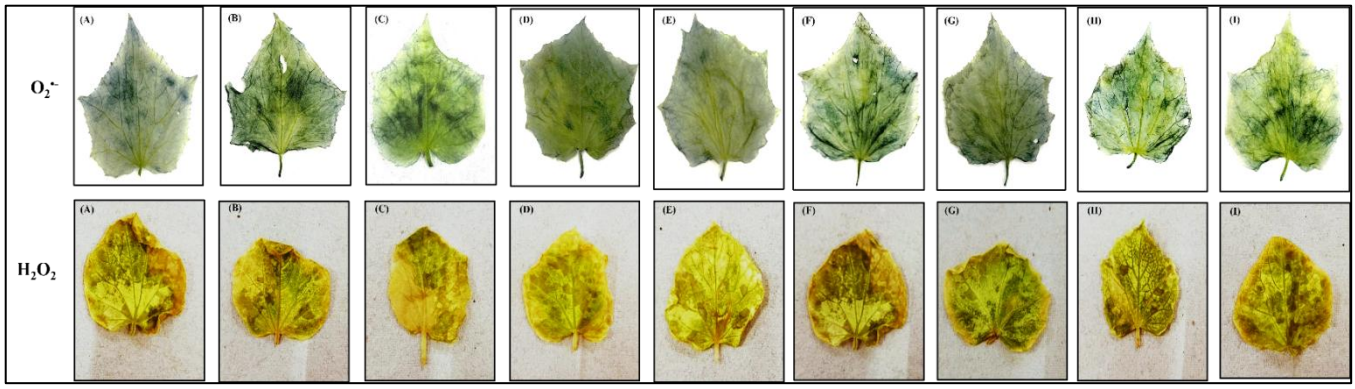


Figure 4.21: Impact of exogenous supplementation of H_2S and NO in-vivo visualization superoxide radicals $O_2^{\bullet-}$ and H_2O_2 in cucumber seedlings as visualized through NBT and DAB staining under (A) Control (without treatment), (B) NaCl, (C) NaCl + NaHS, (D) NaCl + SNP, (E) NaCl + NaHS+ SNP, (F) NaCl + NaHS + L-NAME, (G) NaCl + SNP + L-NAME, (H) NaCl + NaHS+cPTIO and (I) NaCl + SNP + cPTIO

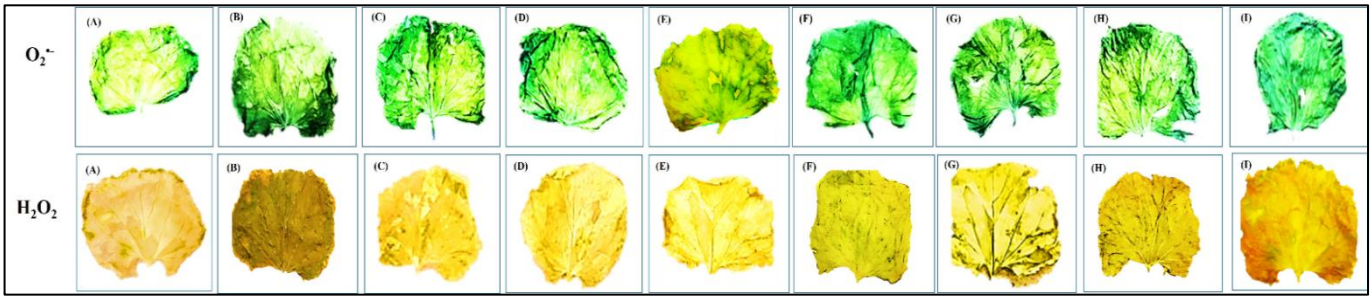


Figure 4.22: Impact of exogenous supplementation of H_2S and NO in-vivo visualization superoxide radicals $O_2^{\bullet-}$ and H_2O_2 in bitter gourd seedlings as visualized through NBT and DAB staining under (A) Control (without treatment), (B) NaCl, (C) NaCl + NaHS, (D) NaCl + SNP, (E) NaCl + NaHS+ SNP, (F) NaCl + NaHS + L-NAME, (G) NaCl + SNP + L-NAME, (H) NaCl + NaHS+cPTIO and (I) NaCl + SNP + cPTIO

4.6 Effect on antioxidant defense system

4.6.1 Enzymatic antioxidants

(a) Superoxide dismutase (SOD) activity:

The results regarding SOD activity are presented in **Figure 4.23.** Exposure to NaCl significantly increased SOD levels, with enhancements by 10% in cucumber seedlings. The exogenous application of NaHS and SNP mitigated the salt stress, resulting in further upregulation of SOD activity by 18% and 31% respectively. Additionally, application of NaHS and SNP in combination further increased the SOD activity by 61% as compared to salt stress. Conversely, the use of cPTIO reduced SOD activity by 5% and 26% in NaHS+ cPTIO and

SNP+ cPTIO in cucumber seedlings. Similarly, the application of L-NAME led to a decline in SOD activity by 15% and 39% in NaHS+ L-NAME and SNP+ L-NAME in cucumber seedlings under salt stress, respectively as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

The results in **Figure 4.24** show that NaCl exposure increased SOD activity by 22% in bitter gourd seedlings. Applying NaHS and SNP reduced the salt stress, boosting SOD activity by 8% and 19% respectively as compared to stress. When used together, NaHS and SNP further increased SOD activity by 49% as compared to salt stressed cucumber seedlings. On the other hand, cPTIO reduced SOD activity by 7.2% in NaHS+ cPTIO and 10% in SNP+ cPTIO under salt stress. Similarly, L-NAME decreased SOD activity by 3% in NaHS+ L-NAME and 8% in SNP+L-NAME in bitter gourd seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

(b) Catalase (CAT) activity:

The results pertaining to the CAT activity is depicted in **Figure 4.23.** The CAT activity was enhanced by 53 % under the exposure of NaCl and this activity was further stimulated by 62%, 106% and 139% by NaHS, SNP and NaHS + SNP in salt stressed cucumber seedlings as compared to salt stress. The application of cPTIO decreased the CAT activity by 25% in NaCl+NaHS+ cPTIO and by 50% in NaCl+SNP+ cPTIO in cucumber seedlings. Under the application of L-NAME, the CAT activity declined by 19% and 25% respectively in NaCl+NaHS+ L-NAME and NaCl+SNP+ L-NAME compared to NaCl+ NaHS and NaCl+ SNP treated cucumber seedlings.

The results for CAT activity, shown in **Figure 4.24,** indicate an increase by 44% under NaCl exposure in bitter gourd seedlings. This activity was further enhanced by the application of NaHS, SNP, and their combination, with an increase by25%, 41%, and 86%, respectively, as compared to salt stress. However, CAT activity decreased by 8% in NaCl+NaHS+ L-NAME and by 24% in NaCl+ SNP+L-NAME compared to NaCl+ NaHS and NaCl+ SNP treated seedlings. In given seedlings, cPTIO reduced CAT activity by 16% in NaCl+ NaHS+ cPTIO and by 59% in NaCl+ SNP+ cPTIO.

(c) Glutathione-S-transferase (GST) activity:

The results for GST activity, presented in **Figure 4.23**, show a 14% increase under NaCl exposure in cucumber seedlings. The application of NaHS, SNP, NaHS+ SNP further boosted the GST activity by 15%, 26%, and 46%, respectively, as compared to salt stressed cucumber seedlings. However, GST activity decreased by 12% in NaCl+ NaHS+ L-NAME and 24% in NaCl+ SNP+L-NAME under salt stress. Additionally, in the seedlings treated with cPTIO, GST

activity dropped by 17% in NaCl+ NaHS+ cPTIO and 43% in NaCl+ SNP+ cPTIO compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

The GST activity results, as illustrated in **Figure 4.24**, indicate a 13% increase in bitter gourd seedlings when exposed to NaCl. This was further upregulated by NaCl+NaHS, NaCl+SNP, and the combined application of NaCl+ NaHS+ SNP, resulting in increases by 7%, 15%, and 26%, respectively, compared to salt stressed seedlings. Nevertheless, GST activity experienced by 21% decrease in NaCl+ NaHS+ L-NAME and a 29% decrease in NaCl+SNP+L-NAME when exposed to salinity. Furthermore, GST activity of given seedlings treated with cPTIO decreased by 26% in NaCl+ NaHS+ cPTIO and 41% in NaCl+SNP+ cPTIO compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

(d) Guaiacol peroxidase (GPX):

The GPX activity results, as illustrated in **Figure 4.23**, indicate a 35.8% increase in cucumber seedlings when exposed to NaCl as compared to control. This was further upregulated by NaCl+NaHS, NaCl+SNP, and the combined application of NaCl+ NaHS+ SNP, resulting in increases by 15%, 33%, and 52%, respectively, as compared salt stress. Nevertheless, GPX activity experienced by 1% decrease in NaCl+ NaHS+ L-NAME and 18% decrease in NaCl+ SNP+ L-NAME in comparison to seedling treated with NaCl+ NaHS and NaCl+ SNP. Furthermore, the GPX activity of the seedlings treated with cPTIO decreased by 6% in NaCl+NaHS+ cPTIO and 32% in NaCl+SNP+ cPTIO as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

The GPX activity findings, shown in **Figure 4.24**, show a 23% increase in bitter gourd seedlings when exposed to NaCl. This was further elevated by NaHS, SNP, and NaHS+ SNP, resulting in increases by 20%, 31%, and 46%, respectively, as compared to salt stress. Nonetheless, salinity reduced GPX activity by 10% in NaCl+ NaHS+ L-NAME and 21% in NaCl+SNP+ L-NAME. Furthermore, GPX activity of cPTIO-treated seedlings fell by 23% in NaCl+ NaHS+ cPTIO and 40% in NaCl+SNP+ cPTIO compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

4.6.2 Enzymes of ascorbate-glutathione (AsA-GSH) cycle:

(a) Ascorbate peroxidase (APX) activity:

The results regarding APX activity are presented in **Figure 4.23.** Exposure to NaCl significantly increased APX levels, with enhancements by 66% in cucumber seedlings as compared to control. Moreover, the application of NaHS and SNP also upregulated the APX activity by 35% and 55% respectively as compared to stress. Additionally, the application of NaHS and SNP in combination further increased the APX activity by 87% under salt stress.

Conversely, the use of cPTIO reduced APX activity by 2% and 33% in NaCl+NaHS+ cPTIO and NaCl+SNP+ cPTIO as compared to NaCl+ NaHS and NaCl+ SNP treated cucumber seedlings. Additionally, the application of L-NAME led to a decline in APX activity by 16% and 14% in NaCl+NaHS+ L-NAME and NaCl+ SNP+ L-NAME compared to NaCl+ NaHS and NaCl+ SNP treated cucumber seedlings under salt stress, respectively.

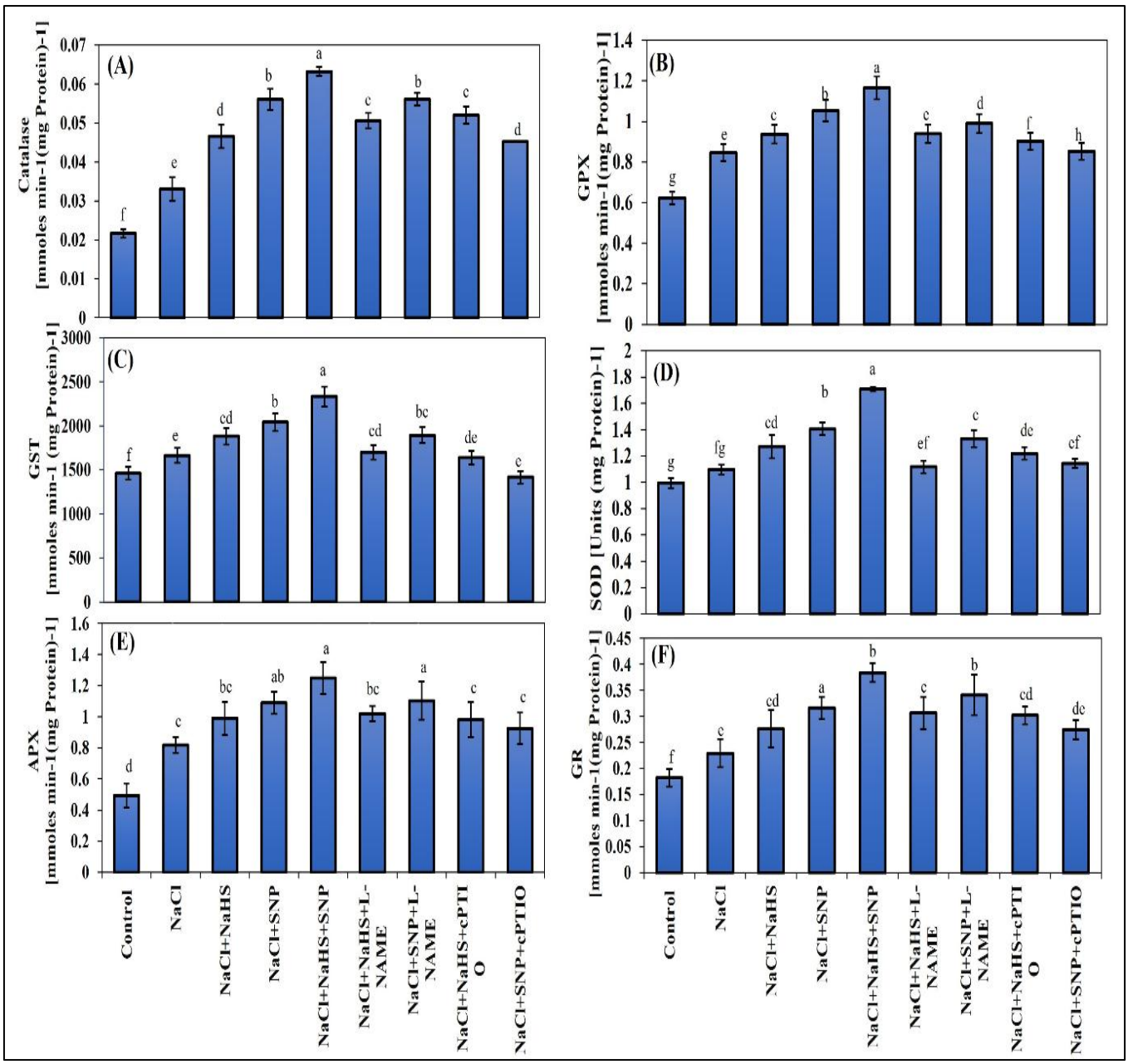


Figure 4.23: The effects of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) through a nutrient solution on Catalase (A), GPX (B), GST (C), SOD (D), APX (E), GR (F) in salt-stressed cucumber seedlings. The results presented are the means ± standard error of three replicates (n = 3). The DMRT (Duncan Multiple Range Test) analysis of the one-way ANOVA indicates that the letters exhibit a significant difference with a p-value of less than 0.05.

Figure 4.24, show that NaCl exposure increased APX activity by 41% as compared to control in bitter gourd seedlings. Furthermore, applying NaHS and SNP also boosted APX activity by 28% and 50%, respectively as compared to stress. When used together, NaHS and SNP further increased APX activity by 90% under salt stress. On the other hand, cPTIO reduced APX activity by 25% in NaCl+ NaHS+ cPTIO and 3% in NaCl+ SNP+ cPTIO under salt stress. Similarly, L-NAME decreased APX activity by 15% in NaCl+ NaHS+ L-NAME and 7% in NaCl+ SNP+L-NAME in bitter gourd seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated bitter gourd seedlings.

(b) Glutathione reductase (GR) activity:

The results for GR activity are shown in **Figure 4.23,** NaCl exposure significantly enhanced GR levels in cucumber seedlings by 25%. The application of NaCl+ NaHS and NaCl+ SNP also increased GR activity by 26% and 48%, respectively as compared to stress. Furthermore, combining NaCl+ NaHS+ SNP increased GR activity by 85% as compared to salt stress. On the other hand, when NaCl+ NaHS+ L-NAME and NaCl+ SNP+ L-NAME were applied to cucumber seedlings under salt stress, GR activity decreased by 16% and 5%, respectively as compared to NaCl+ NaHS and NaCl+ SNP treated bitter gourd seedlings. In contrast, cPTIO reduced GR activity under salt stress by 14% and 23% respectively in NaCl+ NaHS+ cPTIO and NaCl+ SNP+ cPTIO treated seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated cucumber seedlings.

The results in **Figure 4.24,** reveal that NaCl treatment raised GR activity in bitter gourd seedlings by 68% compared to control. Moreover, administering NaCl+ NaHS and NaCl+ SNP to seedlings increased GR activity by 15% and 57%, respectively as compared to stress. When taken combinedly, NaCl+ NaHS and SNP boosted GR activity by 75% as compared to salt stress. Under salt stress, cPTIO reduced APX activity by 37% in NaCl+ NaHS+ cPTIO and 93% in NaCl+ SNP+ cPTIO, respectively. Similarly, L-NAME reduced GR activity by 6.2% in NaCl+ NaHS+ L-NAME and 49% in NaCl+ SNP+L-NAME in bitter gourd seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings.

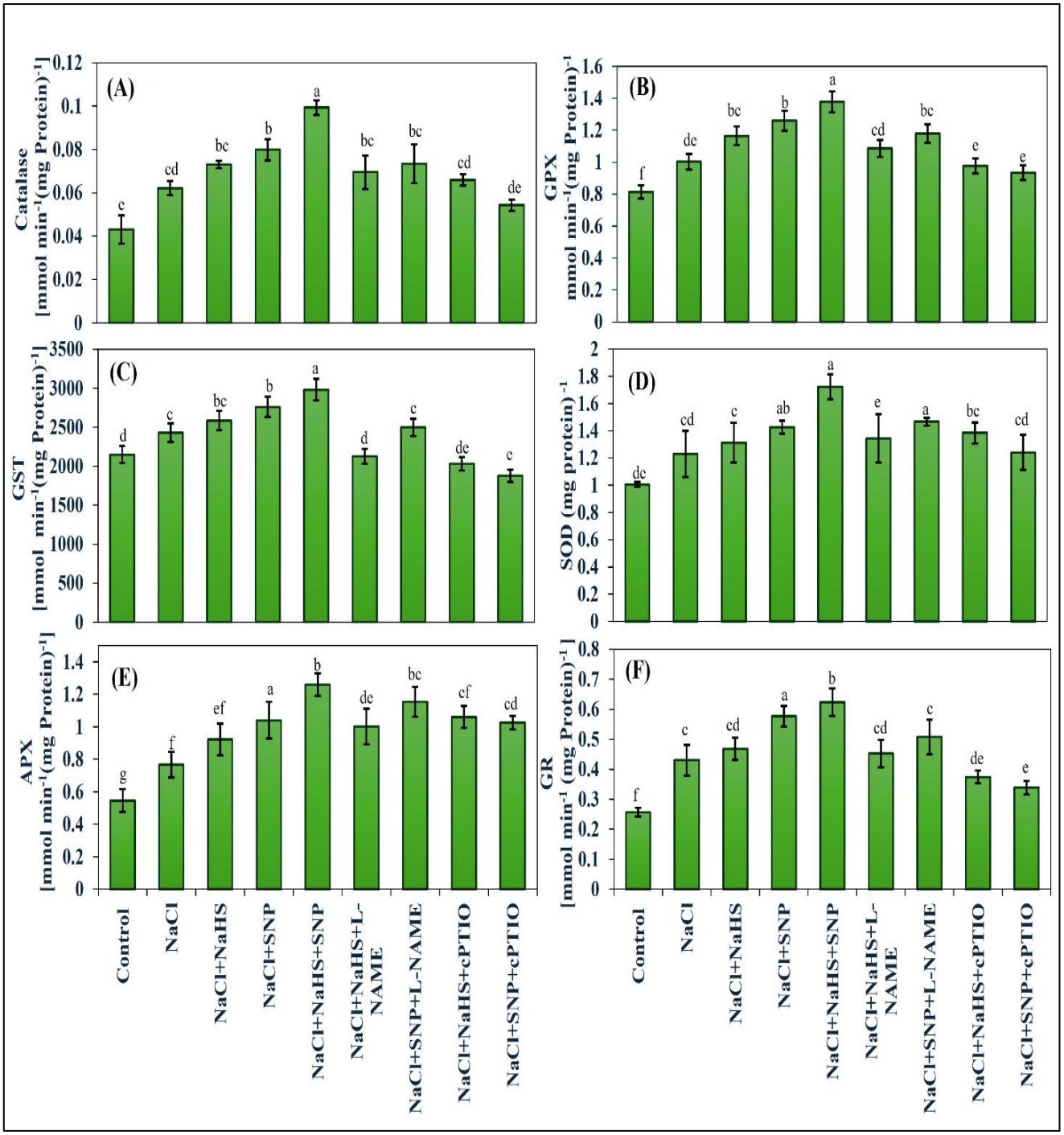
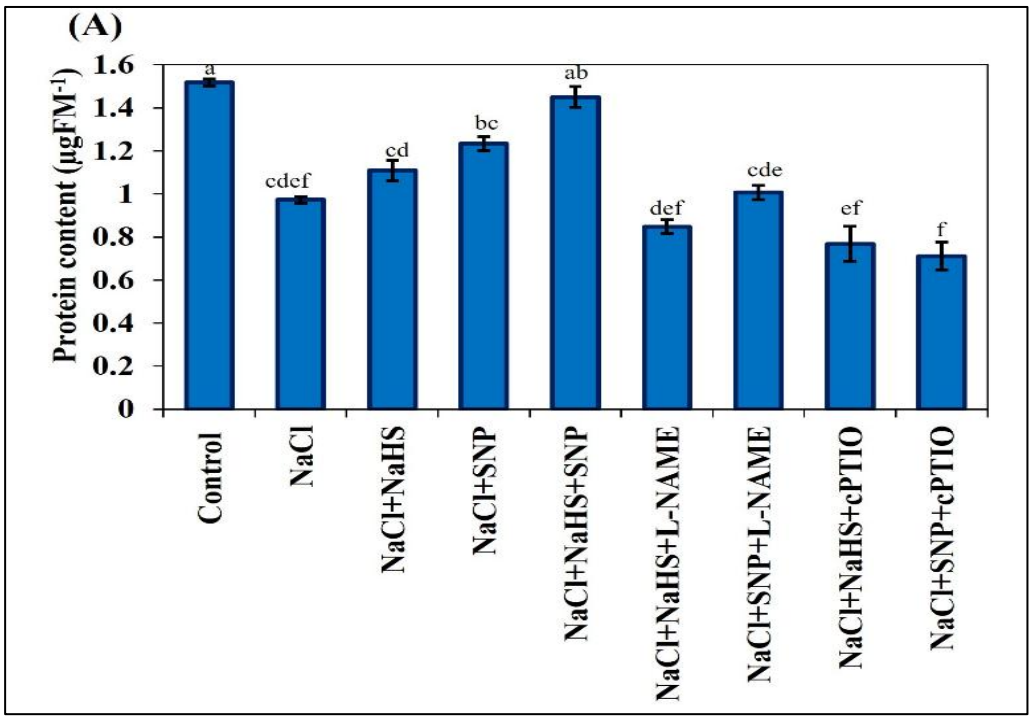


Figure 4.24: The effects of sodium hydrogen sulphide (NaHS) alone or in combination with sodium nitroprusside (SNP; donor of NO), L-NAME (inhibitor of NO), and cPTIO (scavenger of NO) through a nutrient solution on Catalase (A), GPX (B), GST (C), SOD (D), APX (E), GR (F) in salt-stressed bitter gourd seedlings. The results presented are the means + standard error of three replicates (n=3). The DMRT (Duncan Multiple Range Test) analysis of the one-way ANOVA. Mean value represented as bars with different letters for each attribute indicate a significant difference ($P< 0.05$).

4.7 Protein Content:

Figure 4.25 illustrates the findings concerning protein content. Under NaCl duress, the protein content of cucumber and bitter gourd seedlings decreased by 36% and 43% as compared to control. Additionally, in cucumber seedlings, the protein content was elevated by 9% and 17% because of the application of NaHS and/or SNP individually. However, when these stressed seedlings were subjected to NaHS + SNP in combination, the protein content further elevated by 31% as compared to salt stress. Similarly, the protein content in bitter gourd seedlings was elevated by 15% and 26% because of the individual administration of NaHS and SNP and this elevation was further increased by 44 % in the combined NaHS and SNP treated seedlings highlighting the synergistic effect of these treatments in promoting protein synthesis as compared to salt stress.

To gain a more comprehensive understanding, the seedlings were exposed to cPTIO, resulting in a significant decrease in protein content by 22% in NaCl+ NaHS+ cPTIO and by 34% in NaCl+ SNP+ cPTIO treated cucumber seedlings and by 4% in NaCl+NaHS+ cPTIO and 20% in NaCl+SNP+ cPTIO under salt stress in bitter gourd seedlings as compared to NaCl+ NaHS and NaCl+ SNP treated seedlings respectively. In addition, the protein degradation was reduced by the addition of L-NAME by 17% in NaCl+ NaHS+ L-NAME and 25% in NaCl+ SNP+ L-NAME, cucumber seedlings and by 9 % by the addition of L-NAME in NaCl+ NaHS+ L-NAME and by 20% in NaCl+ SNP+ L-NAME respectively compared to NaCl+ NaHS and NaCl+ SNP treated cucumber seedlings.



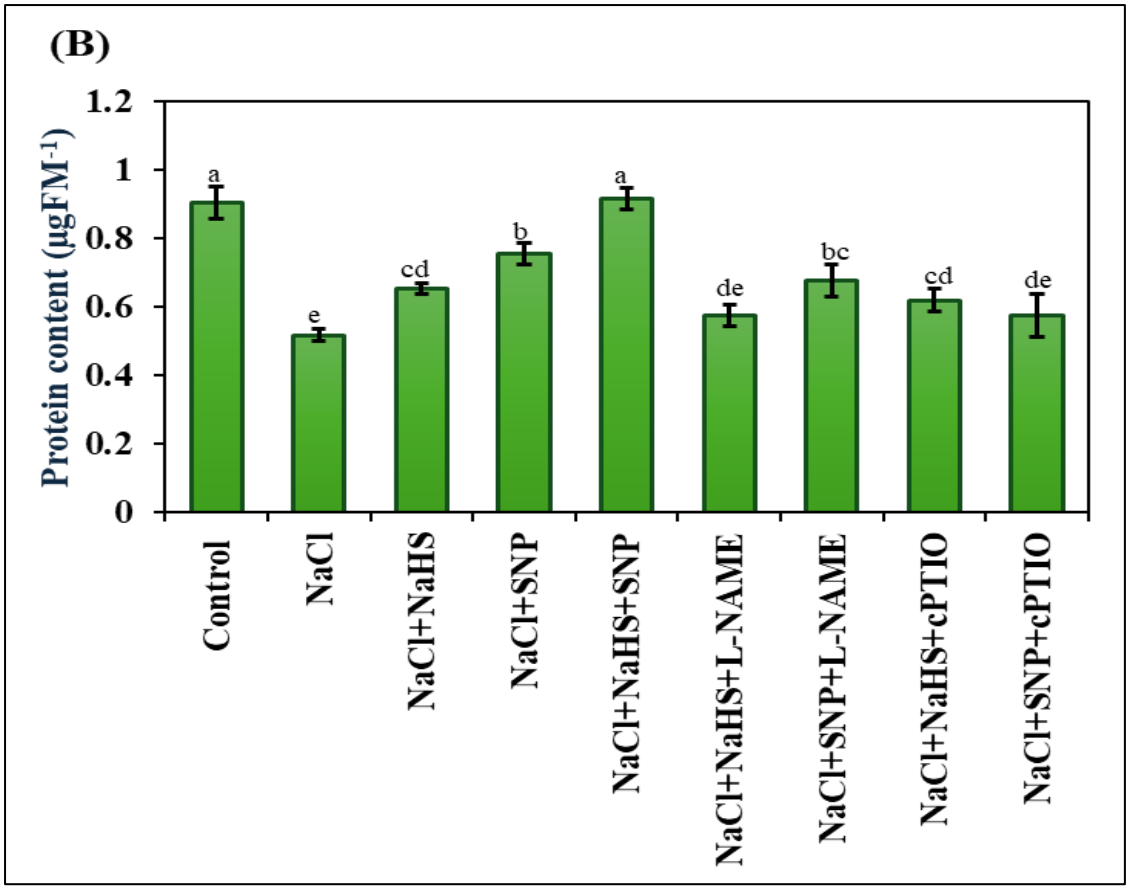


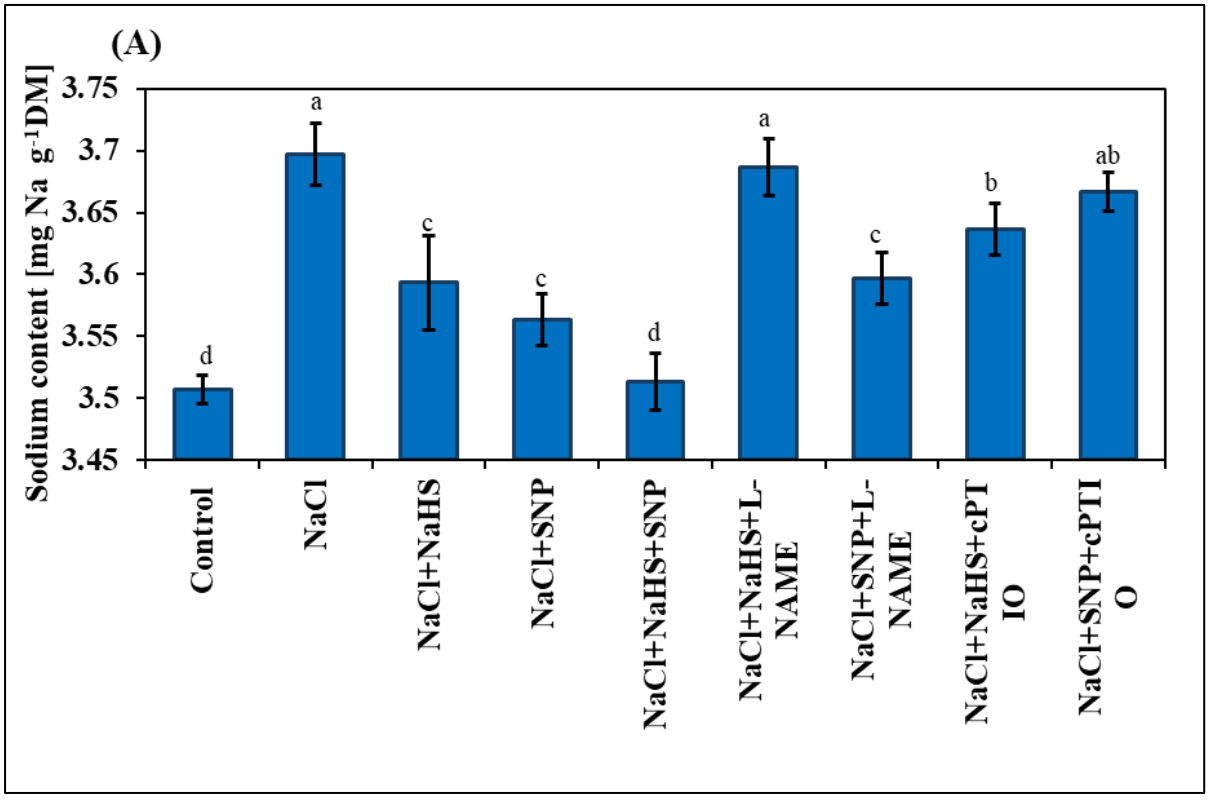
Figure 4.25: Protein content on fresh weight (FW) basis parameters in salt-stressed cucumber (A) and bitter gourd (b) seedlings supplied with sodium hydrogen sulfide (NaHS) alone or together with sodium nitroprusside (SNP; donor of NO), L-NAME (in inhibitor of NO) and cPTIO, scavenger of NO through nutrient solution. The results presented are the means ± standard error of three replicates (n = 3). The DMRT (Duncan Multiple Range Test) analysis of the one-way ANOVA indicates that the letters exhibit a significant difference with a p-value of less than 0.05

4.8 Mineral content (Na^+ and K^+):

In this study, the effect of NaCl, H_2S, NO, cPTIO, and L-NAME in modulating the Na^+ and K^+ contents in cucumber and bitter gourd seedlings under salt stress were also elucidated, highlighting their critical impacts on ionic homeostasis, a vital component of salt tolerance mechanisms. In cucumber seedlings, Na^+ content increased by 5.4%, while K^+ content decreased by 13% under NaCl stress compared to control. Similarly, in bitter gourd seedlings, Na^+ content increased by 4.5%, and K^+ content decreased by 12%, as compared to control.

Supplementation with NaHS (NaCl + NaHS), SNP (NaCl + SNP), and the combination of both (NaCl + NaHS + SNP) significantly alleviated these effects, restoring ionic balance (**Figure 4.26 and 4.27).** In cucumber, NaCl + NaHS treatment reduced Na^+ content by 3% and increased K^+ content by 8% compared to NaCl-treated seedlings. The NaCl + SNP treatment reduced Na^+ content by 3.8% and increased K^+ content by 8%. The combined NaCl + NaHS + SNP treatment was the most effective, reducing Na^+ content by 5.22% and increasing K^+ content by 11.3% as compared to stress. In bitter gourd seedlings, NaCl +NaHS treatment

reduced Na^+ by 2.5 % and increased K^+ by 12%, while NaCl + SNP treatment reduced Na^+ by 3.4% and increased K^+ by 6%. The NaCl + NaHS + SNP combination further reduced Na^+ content by 4.8% and increased K^+ content by 11%, emphasizing its potential to enhance salt tolerance in both seedlings as compared to NaCl.



Conversely, the application of L-NAME and cPTIO negatively impacted Na^+ and K^+ homeostasis under NaCl stress. In cucumber seedlings, L-NAME treatment with NaHS and SNP caused 2.66% and 1% increase in Na^+ content and a 11% and 6% decrease in K^+ content, while cPTIO treatment resulted in 1.24% and 3% increase in Na^+ content and 6% and 12% decrease in K^+ content as compared to NaCl + NaHS and NaCl + SNP seedlings. caused an increase by 0.82% and 2.6% in Na^+ and by 5.4% and 11% decrease in K^+ content as compared to NaCl + NaHS and NaCl + SNP treated seedling under salt stress.

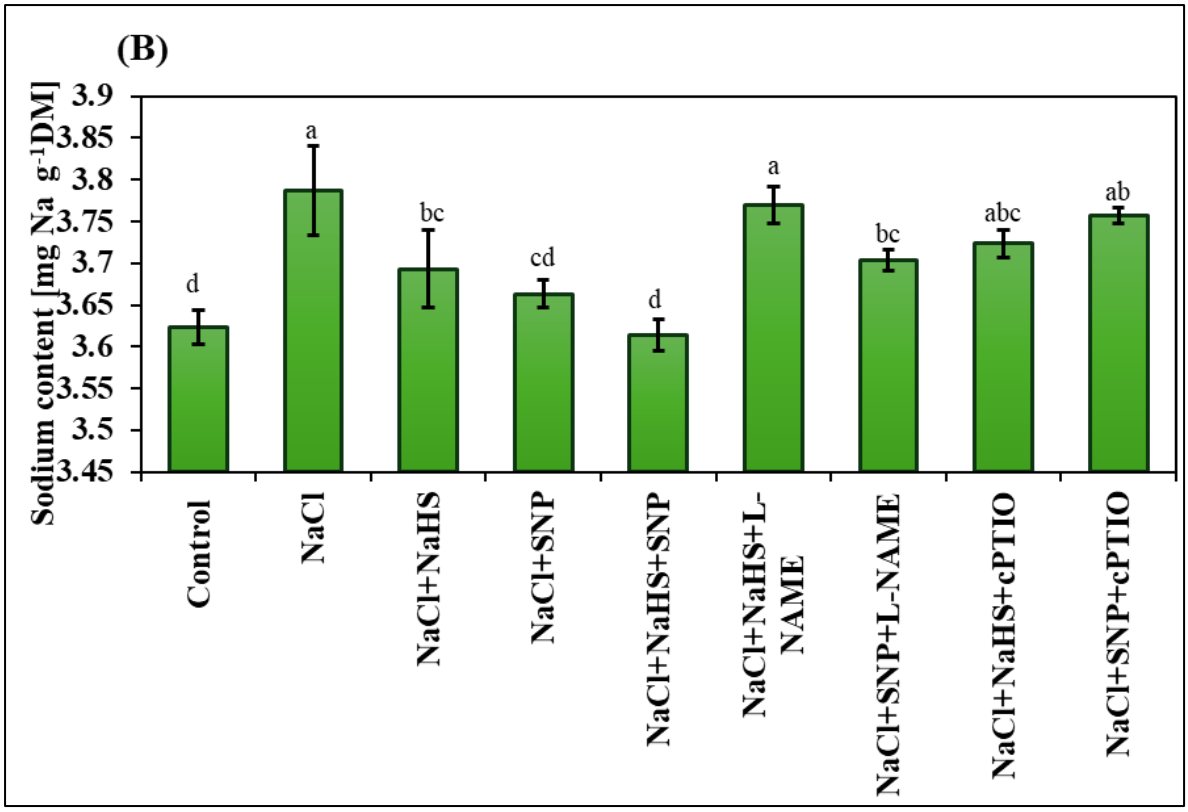
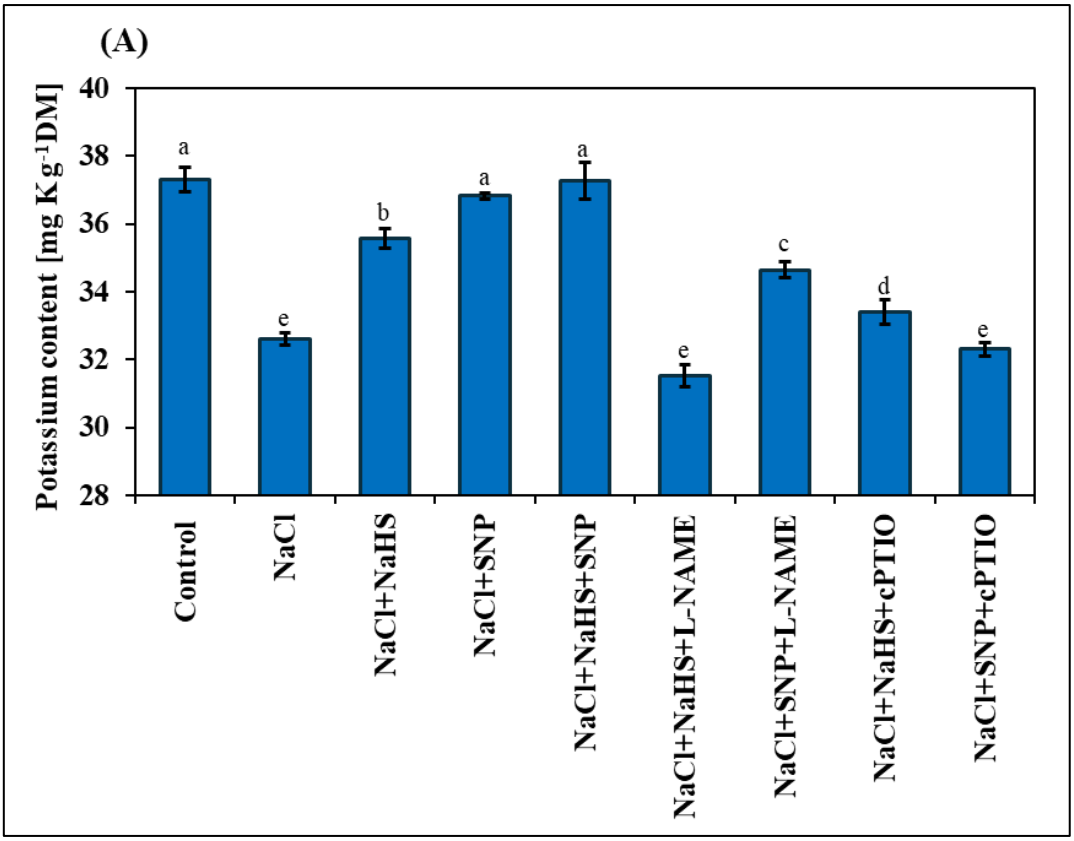


Figure 4.26: The impact of external supplementation of sodium hydrogen sulfide (NaHS) alone or together with sodium nitroprusside (SNP; donor of NO), L-NAME (in inhibitor of NO) and cPTIO, (scavenger of NO) on sodium content in cucumber (A) and bitter gourd (B) seedlings under NaCl stress. Results are expressed as means ± standard error of three replicates (n=3). Different letters assigned to the bars indicate significant differences at $p \leq 0.05$, determined by Duncan's analysis of one-way ANOVA.

On the other hand, in bitter gourd seedlings, application of L-NAME with NaHS and SNP treatment led to an increase by 2% and 1% in Na^+ content and 10% and 6 % decrease in K^+ content compared to NaCl + NaHS and NaCl + SNP treated seedling under salt stress. Likewise, cPTIO treatment.


(A)
Potassium content [mg K g-1DM]
40
38
36
34
32
30
28
a
e
b
a
a
e
c
d
e
Control
NaCl
NaCl+NaHS
NaCl+SNP
NaCl+NaHS+SNP
NaCl+NaHS+L-NAME
NaCl+SNP+L-NAME
NaCl+NaHS+cPTIO
NaCl+SNP+cPTIO

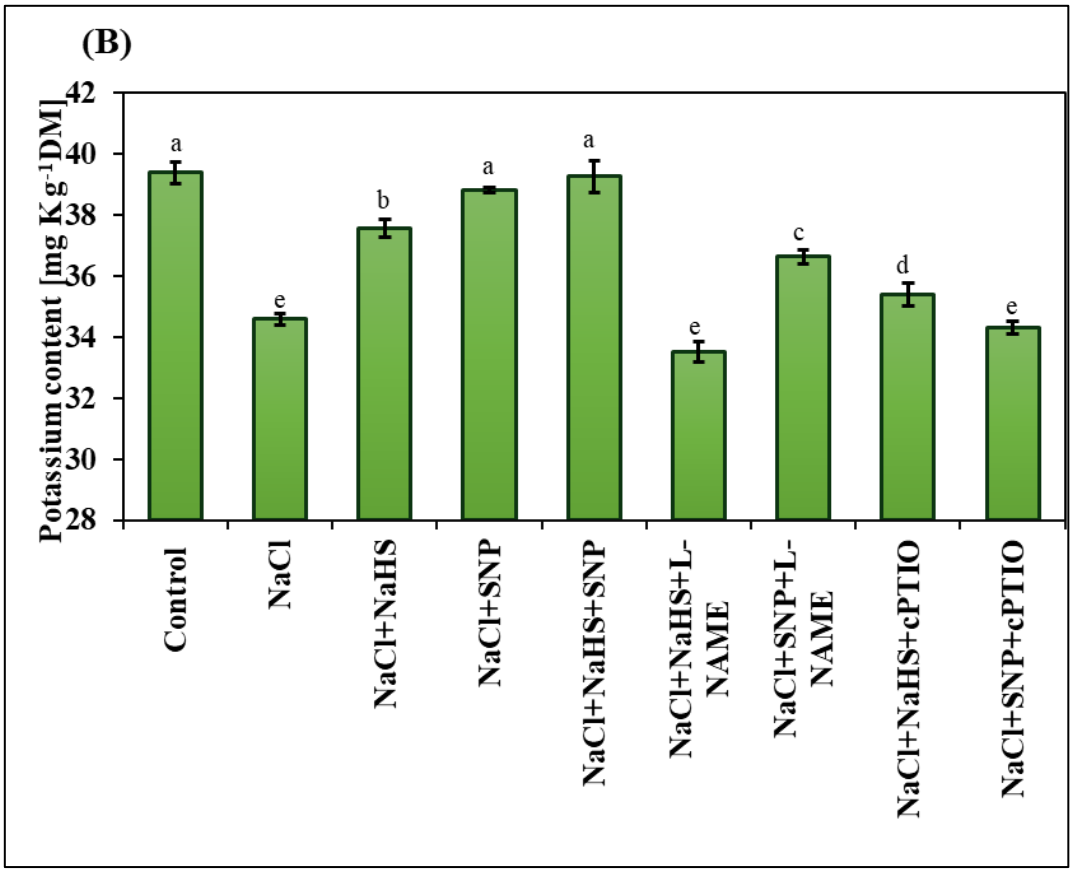

(B)
Potassium content [mg K g-1DM]
42
40
38
36
34
32
30
28
a
e
b
a
a
e
c
d
e
Control
NaCl
NaCl+NaHS
NaCl+SNP
NaCl+NaHS+SNP
NaCl+NaHS+L-NAME
NaCl+SNP+L-NAME
NaCl+NaHS+cPTIO
NaCl+SNP+cPTIO

Figure 4.27: The impact of external supplementation of sodium hydrogen sulfide (NaHS) alone or together with sodium nitroprusside (SNP; donor of NO), L-NAME (in inhibitor of NO) and cPTIO, (scavenger of NO) on potassium content in cucumber (A) and bitter gourd (B) seedlings under NaCl stress. Results are expressed as means ± standard error of three replicates (n=3). Different letters assigned to the bars indicate significant differences at $p \leq 0.05$, determined by Duncan's analysis of one-way ANOVA.

Combined summary of the effect of NO and H_2S supplementation along with the NO inhibitor and scavenger on the morphological and biochemical parameters is depicted in **Figure 4.28.**

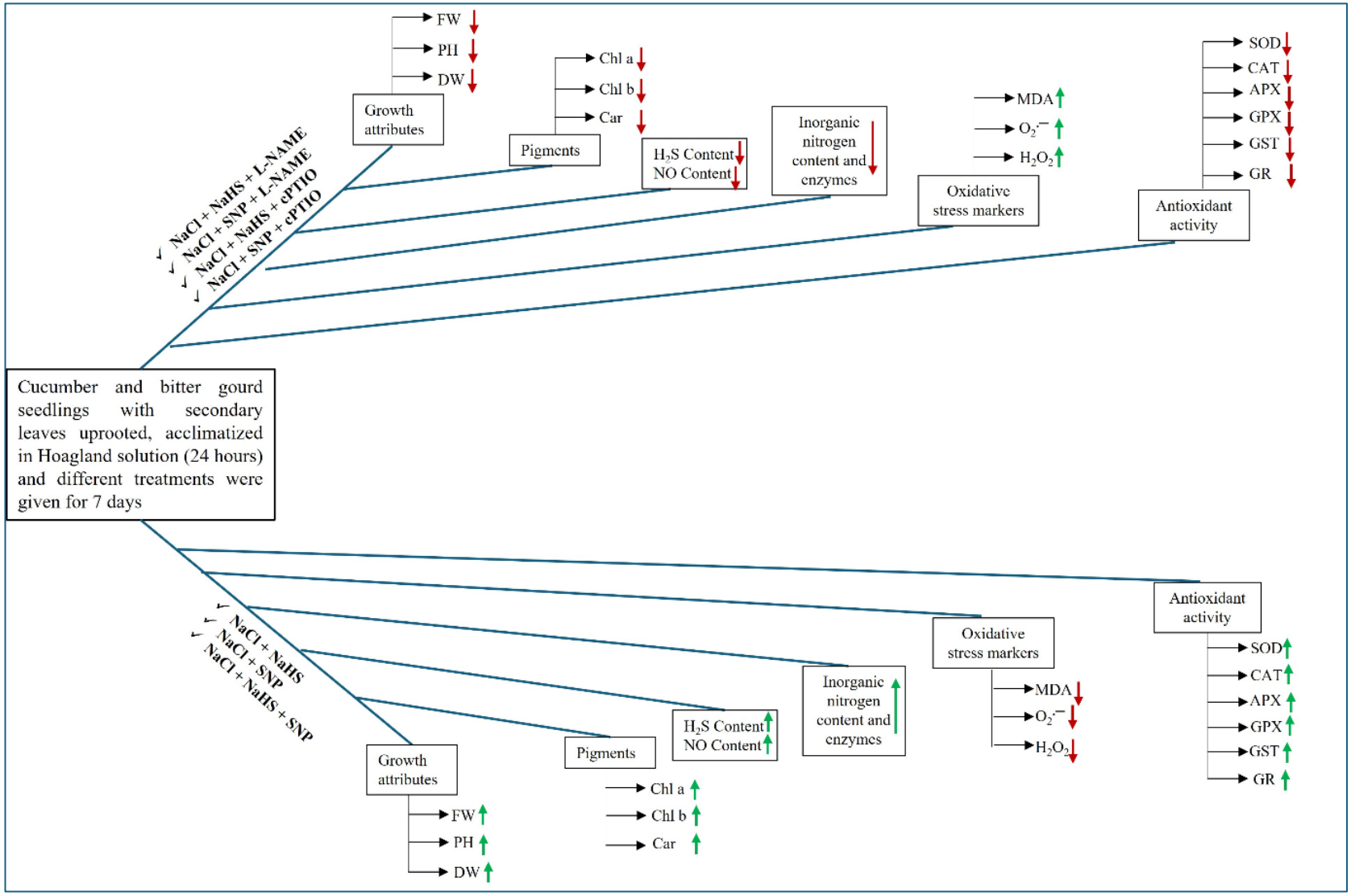


Figure 4.28: A schematic depiction of the biochemical and morphological changes in cucumber and bitter gourdseedlings under the influence of signalling molecules and inhibitors/scavengers of NO used in different combination treatments. Green coloured arrows show the upregulation; red coloured arrows show the downregulation of biochemical and morphological changes.

CHAPTER 5

DISCUSSION

Both NO and H_2S are potential signaling molecules that consists a critical role in regulating plant metabolic activities in challenging circumstances (Antoniou *et al.,* 2020). Previous studies have examined a positive impact of NO and H_2S on plants suffering from salt stress (J. Chen *et al.,* 2015c; Jiang *et al.,* 2019; Kumari *et al.,* 2024,2025). However, insufficient research currently documents the interaction of NO and H_2S to enable the plants to combat the harsh salt stress conditions. This study, therefore aimed at studying the physiological, morphological, and biochemical parameters in cucumber and bitter gourd seedlings exposed to salt stress under the impact of NO and/or H_2S either alone or in combination.

4.1 Regulation of growth attributes by NaHS and SNP under salinity

In this study, the effects of NaCl, NaHS, SNP, L-NAME, and cPTIO in controlling the response of cucumber and bitter gourd seedlings to salt stress were critically analyzed, demonstrating their diverse impacts on plant growth and stress tolerance mechanisms. Sodium chloride induces stress, leading to osmotic imbalances and ionic toxicity, impairing nutrient uptake and cellular functions (Fatima *et al.,* 2022b). These detrimental effects are known to reduce fresh and dry weight and plant height of cucumber and bitter gourd seedlings as evident by the decreased values for these parameters under salt treatment **(Figure 4.1-4.8)**. However, the treatment of NaHS and SNP individually reduced toxicity of NaCl in cucumber and bitter gourd seedlings resulting in increased values for these parameters. This affirms the earlier reports where the application of exogenous H_2S and NO donors resulted in a decrease of the contrary effects of NaCl toxicity on growth attributes (FW, PH, and DW) in tomato and wheat seedlings (Kaya *et al.,* 2023b; Raju & Prasad, 2021a). H_2S is essential for the synthesis of different amino acids and proteins implicated in the enhancement of growth parameters under salt stress (Maryum *et al.,* 2022). Additionally, NO is involved in the development and elongation of roots, differentiation, stem elongation and branching, cell division and expansion (Lamattina *et al.,* 2004). Furthermore, application of H_2S and NO in combination further minimized the harmful impact of salt stress on FW and PH in cucumber and bitter gourd seedlings. This indicated that NaHS and SNP work together to recover the growth traits (FW and PH) of different plants under salt stress. On the other hand, cPTIO (a specific scavenger of NO) and L-NAME (a well known inhibitor of NO) reduced the positive effects of NaHS and SNP in cucumber and bitter gourd seedlings under salt stress. Concomitant with this observation, the root and shoot length declined by 17% and 7% respectively by the application of L-NAME in tomato seedlings subjected to Cr (VI) stress in the presence of calcium and sulfur (Singh & Prasad, 2019). In addition, the effect of SNP was reversed by cPTIO in mustard seedlings, resulting in a decline of FW and DW by 42.2% and 33.3%, respectively, under cadmium stress

(Khan *et al.,* 2020b). These observations indicated that both H_2S and NO play a crucial role in facilitating optimal and sustained signaling to mitigate the toxic effect induced by salt stress on the growth parameters of cucumber and bitter gourd seedlings.

4.2 NaHS and SNP mitigate NaCl-induced damaged photosynthetic pigments

The content of different pigments in the plants serves as a dependable parameter to assess the ability of the plant to resist various stressors (Xue *et al.,* 2013). The present study depicting a drastic decline in the pigments corroborates the previous findings, where salinity stress resulted in reduction of chlorophyll a, chlorophyll b, and carotenoids in tomato and brinjal seedlings (Raju & Prasad, 2023). This decrease in the photosynthetic pigments can be associated to the activation of a chlorophyll-degrading enzyme i.e. chlorophyllase under salinity (Gong *et al.,* 2013). The treatment of NaHS and SNP either individually or in combination in cucumber and bitter gourd seedlings mitigated the damaging effects of NaCl on pigment content as evident by their increased values in the present study **(Figure 4.9,4.10)**. H_2S positively influence chlorophyll content by promoting the synthesis of heat shock proteins like ETHE1, -also known as persulfide dioxygenase, and Sucrose nonferme-1, (SNF1-RELATED PROTEIN KINASE2) involved in chloroplast function. Additionally, NO is known to enhance the activity of enzymes like δ-aminolevulinic acid dehydratase, chlorophyll synthase, and Mg-chelatase, which is involved in the synthesis of δ-aminolevulinic acid, a precursor of chlorophyll. The presence of H_2S can enhance the effectiveness of NO in regulating chlorophyll synthesis, thereby ensuring that plants have sufficient chlorophyll for optimal light absorption and photosynthesis (Yang *et al.,* 2022). It has also been postulated that NO and H_2S improve the uptake of essential nutrients like iron and magnesium, which are the cofactors required for chlorophyll and carotenoid biosynthesis. A study conducted in rice seedlings involving NO and H_2S donors has reported a favorable reaction of plants to these signaling molecules under salinity conditions due to their role in increasing the quantity of grana lamellae and the synthesis of chlorophyll pigments (Mostofa *et al.,* 2015; Ji *et al.,* 2019). Furthermore, another study revealed that spraying SNP on both types of turfgrass boosted the levels of chlorophyll *a* and *b* relative to control plants during drought stress (Hatamzadeh *et al.,* 2015). NO has also been reported to have a beneficial impact on the chlorophyll and carotenoid levels, as well as the photosynthetic rate of *T. aestivum* plants under salt stress (Perlikowski *et al.,* 2022; Kaya *et al.,* 2023).

However, the treatment of L-NAME and cPTIO countered the beneficial effects of H_2S and NO by declining the pigment content of cucumber and bitter gourd seedlings. A study by Verma and Prasad, (2021), reported that exogenous application of L-NAME and cPTIO

abolished the positive effect of SNP in cyanobacteria under Cd stress. A recent study conducted in *Nostoc muscorum* to study the role of NO donors, scavengers and inhibitors under UV radiation, documented that not only the addition of SNP but also the addition of ALA (5-aminolevulinic acid; precursor of chlorophyll) enhanced the content of photosynthetic pigments indicating their roles in ameliorating the effects of radiation. However, supplementation with L-NAME and cPTIO reversed the beneficial effects of ALA demonstrating their detrimental impact on *Nostoc muscorum* growth under UV-B stress. This reversal occurred because cPTIO/L-NAME limited the availability of NO, thereby obstructing NO's role in ALA-mediating signalling (Singh *et al.,* 2024).

4.3 Regulation of H_2S and NO content under salinity:

The H_2S and NO content in cucumber and bitter gourd seedlings was significantly affected by salinity and this was significantly reversed upon the application of different signalling molecules. The present study, shows a substantial decline in H_2S and NO content and this is in concordance with previous findings by Kaya *et al.,* (2023a); Raju and Prasad, (2023) which reported a reduction in H_2S and NO content in tomato and wheat seedlings under salinity stress. NaHS and SNP increased the H_2S and NO content under salt stress and it was reversed by the supplementation of inhibitors and scavengers **(Figure 4.11,4.12)**. It is reported that the endogenous levels of H_2S increases due to the enhanced activity of the D/L-cysteine desulfhydrase (CD) enzyme (Jiang Jing Long *et al.,* 2019). According to Chen *et al.,* (2015), the presence of H_2S was found to augment the capacity of barley to withstand salt via regulating ion homeostasis through the involvement of NO. The rise in NO content responsible for the alleviation of salinity stress by H_2S has been related to an elevation in the activity of nitrate reductase (NR) (Ma *et al.,* 2019). Similarly, the exogenous application of H_2S was found to enhance the endogenous synthesis of NO in *Cyclocarya paliurus* (wheel wingnut) and common beans. This improvement in NO production was observed to facilitate plant salt tolerance through a correlated underlying signalling crosstalk mechanism, as reported previously (Chen *et al.,* 2021; Dawood *et al.,* 2022). Several earlier research studies have demonstrated that salt stress increased the level of H_2S in *Medicago sativa* and promoted NO synthesis in rapeseed, which correlated with the findings of the present study (Lai *et al.,* 2014; Zhao *et al.,* 2018). Additionally, in the context of Cr (VI) toxicity, the presence of NO was found to enhance the activity of CD, hence leading to an enhanced synthesis of H_2S in tomato plants (Alamri *et al.,* 2020). A similar study has been observed by Kaya *et al.,* (2020) where the effect of SNP was imitated by NaHS on NO synthesis in wheat plants under cadmium stress.

In contrast L-NAME and cPTIO supplementation shows a drastic declined in the H_2S and NO content. In a similar study, the growth of mustard treated with As and Ca^{2+} was hindered alongside reduced NO accumulation in the presence of L-NAME and cPTIO, indicating that NO is necessary for complete and persistent signaling in the presence of Ca^{2+} (Singh *et al.,* 2020a).

4.4 NaHS and SNP boosts inorganic nitrogen levels and its enzymes activities under stress

Nitrogen (N) is a key nutrient for plants, crucial for processes like growth, protein and nucleic acid formation, and the synthesis of chlorophyll and other essential molecules. The plants primarily use NO_3^- as a nitrogen source, which is converted to NO_2^- and NH_4^+ through the activities of NR and NiR (Balliu, Sallaku, & Rewald, 2015). In this study, salt stress led to reduced uptake of inorganic nitrogen and a reduction in NR and NiR activities in cucumber and bitter gourd seedlings. This reduction is linked to a decline in NO_3^- uptake, likely caused by interference with the ATP-dependent transport process and disruptions in the electron transport chain (ETC), which supplies the ATP needed for this process (Wakeel *et al.,* 2020). Similar findings were observed in other cyanobacteria, luffa, tomato and brinjal under UV-B radiation, salinity and heavy metals stress (Parihar, Singh, Singh, & Prasad, 2021; Raju & Prasad, 2023; Garima Singh *et al.,* 2024).

However, when exogenous NaHS and SNP were added, either separately or together, they helped alleviate nutrient uptake issues and allowed for recovery of NR and NiR activity under stress. In our study, H_2S and NO supplementation significantly increased NO_3^- content and NR activity, potentially due to the enhanced expression of the NR1 gene in NaCl-treated seedlings. This increased NO_3^- uptake not only stimulated NR activity but also enhanced NiR activity, likely due to the abundance of transcription factors associated with the NiR-encoding gene, as evidenced by the elevated NO_2^- content in both test seedlings under NaCl stress. These results align with previous studies showing that H_2S and NO can synergistically regulate nitrogen metabolism in stressed tomato, brinjal and cyanobacteria under Ni stress (Raju and Prasad, 2023; Singh *et al.,* 2024). When cPTIO, L-NAME were added along with NaHS and SNP under stress, there was a significant decrease in the uptake of inorganic nitrogen (such as nitrate and nitrite) and a reduction in the activity of nitrogen-assimilating enzymes like NR and NiR. This reduction highlighted the role of H_2S and NO in regulating nitrogen assimilation at both transcriptional and post-transcriptional levels, as reported by Balotf *et al.,* (2018). These findings are consistent with the study by Nabi *et al.,* (2019) on Ni-stressed rice seedlings, where

similar inhibitory effects were observed upon blocking H_2S and NO signaling. This further supports the idea that H_2S and NO act as regulatory molecules in mitigating nitrogen-related stress responses in both seedlings.

4.5 NaHS and SNP enhances ammonia and its assimilating ezymatic activities under stress:

Ammonium ions (NH_4^+) are the final products of nitrate assimilation, but due to their toxicity, they must be either quickly removed or incorporated into organic compounds. In cucumber and bitter gourd seedlings, NH_4^+ is primarily assimilated through the GS-GOGAT pathway, where GS and GOGAT convert ammonium into glutamine and glutamate, essential for cellular functions. In this study, salinity significantly reduced GS and GOGAT activities **(Figure 4.17, 4.18),** likely due to increased NH_4^+ accumulation, disruption of the electron transport chain (ETC) leading to lower ATP synthesis, and osmotic and pH imbalances affecting growth and photosynthesis. Similar declines have been reported in Cd-stressed *Nostoc muscorum* and Ni-stressed *Anabaena sp.* (Ahad *et al.,* 2017; Verma & Prasad, 2021). However, exogenous NaHS + SNP, significantly enhanced GS and GOGAT activity, suggesting that H_2S and NO help restore normal ammonium assimilation. These findings align with Balotf *et al.,* (2018), who reported that exogenic SNP accelerated GS and GOGAT activity and alleviated ammonium toxicity in wheat seedlings. Furthermore, Raju and Prasad, (2023), confirmed that H_2S alleviates salt stress and enhances the activity of NH_4^+ assimilation enzymes in tomato and brinjal seedlings.

Additionally, when inhibitors such as cPTIO, L-NAME were applied alongside NaHS and SNP under stress, GS and GOGAT activities were suppressed drastically. This indicates a strong interconnection between H_2S and NO in regulating ammonium detoxification, as blocking their signaling disrupts nitrogen metabolism. Similar results were observed in *Nostoc muscorum* when cPTIO and L-NAME were externally supplied under Ni stress (Verma & Prasad, 2021). Thus, the study highlights the synergistic role of H_2S and NO in mitigating NH_4^+ toxicity and maintaining nitrogen balance in given seedlings under salt stress.

4.5 NaHS and SNP recovers NaCl-induced oxidative stress

Plant's primary stress responses produce ROS, which can cause lipid peroxidation and oxidative damage to the cell (Yin *et al.,* 2016). The amount of $O_2^{\bullet-}$, H_2O_2, and MDA equivalents in the leaves of cucumber and bitter gourd seedlings was measured through biochemical analysis and this was further validated by *in-vivo* visualization of oxidative stress biomarkers

(Figure 4.21, 4.22). The cellular integrity and functions were compromised by the increased H_2O_2 and MDA levels under NaCl treatment, which was attributed to the increased ROS production and lipid peroxidation (Tahjib-Ul-Arif *et al.,* 2018). This is in accordance with the study of Raju and Prasad (2021) which showed that H_2O_2, $O_2^{\bullet -}$ and MDA content was raised considerably around 80%, 63% and 64% under salt stress in tomato seedlings. In the current study, exogeneous application of H_2S and NO decreased the H_2O_2, $O_2^{\bullet -}$ and MDA levels by diminishing ROS generation. Concomitant with these findings, a study conducted by Chen, Chen and Jiang, (2017) in the rice seedlings under mercuric stress reiterated that both H_2S and NO help to lessen the severity of stress in the seedlings by scavenging or limiting the production of oxidative stress. In a mechanistic manner, H_2S increases the activity of antioxidant enzymes, including SOD, CAT, and POD, which are essential for the prevention of oxidative damage and the scavenging of ROS (Siddiqui *et al.,* 2011). The activities of GPX and GR are enhanced by H_2S supplementation, which enhances the synthesis of sulfur-containing compounds such as glutathione. These enzymes are essential for the detoxification of ROS and the maintenance of the cellular redox balance (Arora *et al.,* 2016). NO and H_2S collectively upregulate distinct sets of antioxidant enzymes, providing a more comprehensive defence against oxidative stress.

Conversely, the inhibition of NO signalling by cPTIO and L-NAME reduced the beneficial effects of H_2S, and NO **(Figure 4.19, 4.20),** highlighting the importance of these molecules in mitigating stress. This is supported by a study carried out in tomato subjected to Cr stress which revealed that the addition of cPTIO significantly inhibits the positive effects of NO by increasing the oxidative stress markers (Alamri *et al.,* 2020). Additionally, another study by Li *et al.,* (2017) reported that the simultaneous application of cPTIO and L-NAME increased the MDA content of maize seedlings under drought stress.

4.6 NaHS and SNP maintain redox homeostasis by enhancing the activity of enzymatic antioxidants in seedlings under salinity

In the current work, test seedlings exposed to salt stress showed enhanced activity of SOD, CAT and GST and enzymes of AsA-GSH cycle i.e., APX and GR **(Figure 4.23, 4.24).** Salt stress induces oxidative stress in plants by triggering the overproduction of reactive oxygen species (ROS), leading to cellular damage. Our findings align with prior research, as Ni was previously reported to significantly enhance the activity in studies conducted on *N. muscorum* and *Cucurbita pepo* L. (Valivand *et al.,* 2019; Verma & Prasad, 2021). Additionally, Khan *et al.,* (2020), observed a significant increase in AsA-GSH cycle enzyme activity in *Vigna radiata* seedlings exposed to Cd stress, respectively.

To counteract stress conditions, plants activate a robust antioxidant defense system comprising enzymatic antioxidants such as SOD, CAT, GST, and enzymes of the ascorbate-glutathione (AsA-GSH) cycle, including APX, GR (Cui *et al.,* 2020; Ozfidan-Konakci *et al.,* 2020; Tiwari & Prasad, 2018, 2020). Under salt stress, SOD functions as the primary defense mechanism by dismutating superoxide radicals ($O_2^{\bullet-}$) into hydrogen peroxide (H_2O_2) and oxygen, while CAT, GST, APX, and GR facilitate H_2O_2 detoxification into water and oxygen (Verma & Prasad, 2021). Among these, CAT, containing porphyrin, has a higher turnover rate and primarily initiates detoxification; however, when its activity declines, APX and glutathione peroxidase (GPX) upregulate their enzymatic activity to compensate (Ozfidan-Konakci *et al.,* 2020). Furthermore, cross-tolerance mechanisms suggest that exposure to stress can enhance the antioxidant response to salinity, as observed in our study.

In the current study, NaHS and SNP, either alone or in combination, were also found to up-regulate the antioxidant activities (SOD, CAT, GST, GPX) by reducing the oxidative stress under salinity. Inconclusively, H_2S increases the tolerance of cucumber and bitter gourd seedlings by reducing the absorption of Na^+ and Cl^- ions, maintaining ion homeostasis, enhancing antioxidant potential (Kaya *et al.,* 2023; Sharma *et al.,* 2024). H_2S is also essential for the synthesis of sulphur-containing compounds, such as glutathione, which directly scavenge ROS and regenerate other antioxidants, thereby enhancing the overall antioxidant capacity (Kaur *et al.,* 2023). NO application under saline conditions maintains turgor pressure and protects seedlings, and enhanced the activity of antioxidant enzymes, which helped to diminish membrane permeability, MDA and H_2O_2 content and $O_2^{\bullet-}$ generation. This argument is supported by a study, in which exogenous application of SNP leads to upregulation in the antioxidant activities in mustard seedlings in the presence of arsenic stress Khan *et al.,* (2020). Another study demonstrated that exogenous application of NaHS and SNP resulted in the up-regulation of antioxidant activities in rice seedlings, resulting in improved growth, reduced MDA and ROS levels under NaCl and heat stress (Alamri *et al.,* 2020; Gautam *et al.,* 2022).
On the other hand, suppressing H_2S and NO signaling with cPTIO and L-NAME lowered the beneficial effects of these signaling molecules in reducing oxidative stress. It has been observed by Zhao *et al.,* (2018) that cPTIO scavenged the antioxidant activities of SOD, Catalase, GPX, and GST in rapeseed under salt stress. Furthermore, it was reported that the application of exogenous L-NAME reversed the effect of SNP in bamboo under Cd stress (Emamverdian *et al.,* 2021). It was also observed that after the application of exogenous cPTIO and/or L-NAME, the antioxidative activities dropped as compared to SNP and melatonin application in *Kandelia*

obovate and *Nitraria tangutorum* under cadmium stress and tomato seedlings under salt stress (Hasanuzzaman *et al.,* 2020, Raju *et al.* 2021).

Therefore, these findings indicate that H_2S functions as a downstream signal molecule in activating NO and plays a crucial role in enhancing antioxidant defense mechanisms, regulating H_2S metabolism, and collaborating with NO to alleviate the harmful impacts of salinity on cucumber and bitter gourd seedlings.

4.7 Elucidation of mechanisms with the crosstalk of H_2S and NO

NaCl (salt) stress in plants induces a series of physiological and biochemical disruptions, primarily through osmotic, ionic, and oxidative stresses. Osmotic stress impairs water uptake, creating drought-like conditions, while ionic stress arises from the influx of Na^+ and Cl^- ions, disrupting ion homeostasis and nutrient balance. This ionic imbalance, combined with the toxicity of ROS, leads to oxidative damage at the cellular level. The damage is reflected in reduced photosynthetic efficiency due to loss of pigment content, increased chlorophyllase activity, and damage to the OEC and ETC in PSII and PSI (Khan, Siddiqui, AlSolami, *et al.,* 2020). ROS, such as superoxide and hydrogen peroxide, further exacerbate oxidative stress by modifying proteins, lipids, and membranes. In response, plants activate antioxidant defense mechanisms, but the imbalance between ROS production and detoxification causes ongoing cellular damage. Additionally, salt stress disrupts nitrogen metabolism by reducing nitrate uptake and impairing main enzymes like NR and NiR, leading to ammonium accumulation and a disturbed carbon-to-nitrogen ratio, which inhibits growth. Ammonium accumulation also activates nucleases and proteases, causing membrane instability, lipid peroxidation, and electrolyte leakage (Singh *et al.,* 2024). Furthermore, the inhibition of NiR and the buildup of nitrite exacerbates oxidative damage. The combined effect of NaCl intensifies these disruptions, causing severe damage to proteins, photosynthetic processes, and ROS generation, further hindering plant development and overall health. This multifaceted stress results in nutrient deficiency, metabolic imbalances, and growth inhibition, ultimately limiting the plant's ability to maintain normal physiological functions.

Among key signaling molecules, H_2S and NO play vital roles in modulating plant responses to salinity stress. Both molecules regulate various physiological and biochemical pathways, ensuring ion homeostasis, antioxidant defense, and nutrient assimilation, thereby enhancing plant resilience.

NO is directly involved in maintaining the K^+/Na^+ balance by reducing Na^+ accumulation in the cytosol. It facilitates Na^+ transport into the vacuole (via the tonoplast) or out of the cell (via the plasmalemma) by regulating ion channels and membrane transporters (Fancy, Bahlmann,

& Loake, 2017; Hasanuzzaman *et al.,* 2020). SNP treatment, a NO donor, enhances the K^+/Na^+ ratio under salt stress by activating membrane ion pumps (H^+-ATPase and H^+-PPase) (Fatma, Asgher, Masood, & Khan, 2014). Similarly, H_2S reduces Na^+ and Cl^- uptake, helping plants maintain ion homeostasis (Kaya *et al.,* 2023b; G. Sharma *et al.,* 2024).

NO and H_2S improve photosynthesis by enhancing chlorophyll biosynthesis and stomatal conductance. NO influences chlorophyll synthase and Mg-chelatase, which is crucial for chlorophyll production, while H_2S promotes the synthesis of heat shock proteins like ETHE1 and SNF1, which supports the chloroplast function. Additionally, these molecules improve iron and magnesium uptake, which are the essential cofactors for chlorophyll and carotenoid biosynthesis (Correa-Aragunde *et al.,* 2004; Jiang *et al.,* 2019)

NO and H_2S regulate nitrate assimilation and ammonium metabolism by enhancing NR and NiR activity (Ma *et al.,* 2019). H_2S application increases D/L-cysteine desulfhydrase (CD) activity, leading to elevated endogenous H_2S levels, which in turn stimulate NR expression and NO production. This results in increased NO_3^- content, NR activity, and NiR activity, facilitating nitrogen assimilation. Furthermore, H_2S and NO significantly enhance GS and GOGAT activity, restoring normal ammonium metabolism and supporting plant growth (Maryum *et al.,* 2022). NO and H_2S alleviate oxidative stress by modulating antioxidant enzymes such as SOD, CAT, APX, and GST (Siddiqui *et al.,* 2011). H_2S stimulates the synthesis of sulfur-containing compounds like glutathione, which scavenges ROS and enhances the overall antioxidant capacity (Kaur *et al.,* 2023). Both molecules promote the persulfidation (H_2S-mediated) and S-nitrosylation (NO-mediated) of key proteins, influencing enzyme activities and stress response pathways. For instance, persulfidation of NADPH oxidase at Cys825 and Cys890 enhances ROS signaling for stress adaptation, while S-nitrosylation of APX1 at Cys-32 boosts antioxidant defense (Khan *et al.,* 2019; Wei *et al.,* 2022)

When NO and H_2S signaling is disrupted using inhibitors like cPTIO (which blocks NO) and L-NAME (which inhibits NO synthase), plants exhibit reduced antioxidant activity, increased ROS accumulation, and greater membrane damage. These inhibitors impair ion transport, nitrogen metabolism, and photosynthetic efficiency, with further highlighting the indispensable roles of H_2S and NO in stress mitigation **(Figure 5.1)**.

Together, H_2S and NO orchestrate a complex signaling network that fine-tunes plant responses to salinity stress. Their interplay through redox modifications (persulfidation and S-nitrosylation), ion homeostasis regulation, ROS scavenging, and nitrogen metabolism modulation ensures the physiological stability under adverse conditions. The activation of antioxidant enzymes, enhanced chlorophyll biosynthesis, and improved nutrient uptake which

further contribute to their protective roles. This mechanistic understanding of H_2S-NO crosstalk provides valuable insights into how plants adapt to environmental stresses, offering potential strategies for improving crop resilience to salinity stress.

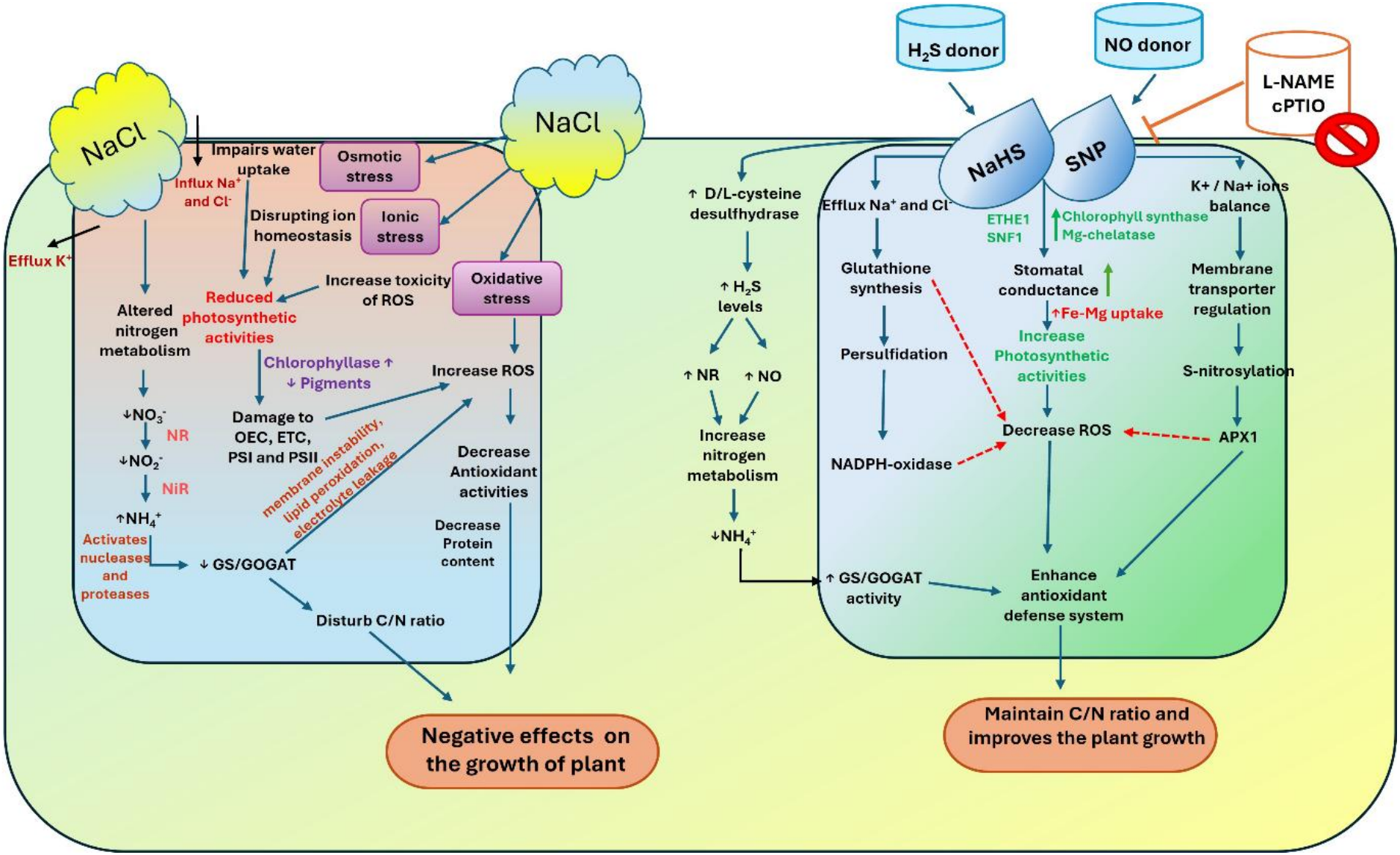


Figure 5.1: Crosstalk of signaling molecules under salt stress in plants highlighting the signaling pathways regulating stress responses, including antioxidant defense, Osmo protection, and gene expression, to enhance salt tolerance.

CHAPTER 6

SUMMARY AND CONCLUSION

Soil salinization, caused by naturally and by humanization, is a major problem in agriculture sector worldwide. High soil salinity disrupts plant metabolism, impairing photosynthesis, enzyme activity, and nutrient uptake while triggering oxidative stress. These issues make it difficult for seeds to germinate, inhibits plant growth, and disrupt photosynthesis, leading to lower crop yields. To cope with salinity, plants adjust hormone levels, activate antioxidant enzymes, and compartmentalize excess salt ions to minimize damage and maintain metabolic balance. However, these natural defenses are often not strong enough to prevent serious damage, requiring extra measures to improve plant tolerance.

Recent research highlights nutrient supplementation, mediated by signaling molecules, as a key strategy to alleviate salt stress. These molecules enhance ion homeostasis, activate stress-responsive pathways, and improve plant resilience. These nutrients help plants to adapt by changing root structure, activating stress-related genes, and increasing antioxidant enzyme activity. For instance, NO assists in alternation of metabolic process, wound healing, UV-B stress response, and coping with high-light irradiance in some species such as *Dasycladus vermicularis, Chlorella pyrenoidosa*, and *Ostreococcus tauri*. Similarly, H_2S shows a central role in different aspects of plant growth, encompassing root architecture, seed germination, stomatal movement, photosynthesis, and the modulation of antioxidant levels under both normal and stressful conditions, provided it is present within appropriate concentrations. The interaction between NO and H_2S in plant signaling under abiotic stress is a fascinating area of research in plant biology and more research is needed to explore how this interaction is imperative to improve plant resilience against salt stress.

The primary objectives of the study were:

1. Analysis of growth pattern and photosynthetic pigments in test seedlings.
2. Analysis of inorganic nitrogen content and co-enzymes involved in nitrate and ammonia assimilation in test seedlings.
3. Evaluation of oxidative damage and antioxidant activity in test seedlings.
4. Elucidation of crosstalk mechanism with nitric oxide.

Methodology:

The seeds of cucumber and bitter gourd were washed, surface-sterilized with 2% NaOCl for 5 minutes, and soaked in distilled water for 2–4 hours. For germination, sterilized seeds were wrapped in muslin cloth and kept in the dark before being planted in sand-filled trays. The seeds were incubated at 25 ± 2°C for 48 hours, then transferred to a growth chamber with controlled light, temperature, and humidity conditions until secondary leaves emerged. Seedlings were then acclimatized in a half-strength Hoagland medium (pH 7.0) for 24 hours.

The experimental design included several combinations (1) control (without treatment), (2) NaCl, (3) NaCl + NaHS, (4) NaCl + SNP, (5) NaCl + NaHS+ SNP, (6) NaCl + NaHS + L-NAME, (7) NaCl + SNP + L-NAME, (8) NaCl + NaHS + cPTIO and (9) NaCl + SNP + cPTIO. Each combination was tested with three replicates, and the experiments were independently repeated three times. Photosynthetic pigments were analysed following the methodology of Lichtenthaler, (1987), whereas NO content and H_2S content was measured by following the methodology of Mostofa *et al.,* (2015) and Zhou *et al.,*(2005). Inorganic nitrogen forms (NO_3^-, NO_2^-, NH_4^+) were also quantified. Enzymatic activities related to nitrogen assimilation, including Nitrate Reductase (Hayaishi *et al.,* 2005), Nitrite Reductase (Losada and Paneque, 1971), Glutamine Synthetase (Cthrine Lillo, 1984), and Glutamate Synthase (Singh and Srivastava,1986), were assessed spectrophotometrically. Sodium (Na^+), potassium (K^+), contents were evaluated according to the method of Kaur, Kaur and Sharma, (2023). Protein content was estimated using the method of Lowry *et al.,* (1951). Oxidative stress markers included H_2O_2 (Alexieva *et al.,* 2001), Superoxide anion content ($O_2^{\cdot-}$) (Elstner and Heupel, 1976) MDA content (Heath and Packer, (1968). *In-vivo* visualization, involved NBT and DAB staining to visualize superoxide (Frahry and Schopfer, 2001).and H_2O_2 accumulation (Thordal-Christensen *et al.,* 1997). Antioxidant enzyme activities, including SOD (Giannopolitis and Ries 1977), Catalase (Barka, 2001), Ascorbate Peroxidase (Barka, 2001), and Glutathione Reductase (Schaedle and Bassham, 1977), Glutathione-S-transferase (Habig and Jakoby, 1981), Glutathione peroxidase (Sharma *et al.,* 2012) were determined spectrophotometrically.

Important Findings:

Salt stress severely impaired plant growth and physiology in cucumber and bitter gourd seedlings, leading to reduced biomass and plant height. It also disrupted photosynthetic efficiency by degrading chlorophyll and carotenoids, impairing nutrient uptake, and destabilizing Na^+/K^+ homeostasis. High Na^+ accumulation caused ionic toxicity, while nitrogen and sulphur metabolism were significantly suppressed, reducing the activity of key enzymes involved in nitrogen assimilation and ammonium detoxification. Furthermore, salt stress triggered oxidative stress, as indicated by increased ROS accumulation and decreased antioxidative potential, leading to cellular damage and protein degradation.

Supplementation with H_2S, and NO (NaCl + NaHS + SNP) effectively alleviated these adverse effects, significantly improving plant growth parameters, restoring chlorophyll and carotenoid levels, and promoting Na^+/K^+ homeostasis by reducing Na^+ toxicity and enhancing K^+ uptake. These treatments enhanced nitrogen metabolism by upregulating key enzymes responsible for nitrogen assimilation and ammonium detoxification, ultimately supporting better stress

adaptation. Additionally, oxidative damage was mitigated through enhanced activity of antioxidant enzymes such as SOD, CAT, APX, GPX, GR and GST, as confirmed by histochemical staining and in vivo visualization of superoxide radicle and H_2O_2 content.

However, the application of cPTIO and L-NAME disrupted these protective effects, leading to higher ROS accumulation, imbalanced Na^+/K^+ levels, and reduced nitrogen metabolism. Plants treated with these inhibitors exhibited increased oxidative stress markers, decreased enzymatic antioxidant activity, and lower protein content, indicating severe cellular damage. The disruption of H_2S and NO signalling hindered stress tolerance mechanisms, reinforcing the crucial role of endogenous H_2S and NO in mitigating salt stress and maintaining redox balance in cucumber and bitter gourd seedlings.

Conclusion

This study demonstrates that salt stress severely hinders the growth, photosynthesis, nutrient balance, and metabolic functions of cucumber and bitter gourd seedlings. However, exogenous supplementation with NaHS (H_2S), and SNP (NO) significantly mitigates these effects by improving plant growth parameters, restoring photosynthetic pigments, maintaining Na^+/K^+ homeostasis, and enhancing nitrogen metabolism. Furthermore, oxidative stress is effectively alleviated through increased antioxidant enzyme activity and NO-mediated ROS detoxification. The combined treatment of NaCl + NaHS + SNP exhibits the most substantial protective effects, highlighting a synergistic role in stress mitigation. Conversely, the inhibition of NO using cPTIO and L-NAME disrupts these protective mechanisms, confirming the essential role of NO in salinity tolerance. These findings emphasize the potential of NaHS (H_2S), and SNP (NO) supplementation in enhancing plant resilience under saline conditions.

CONCLUSION

i. The primary aim of this research was to investigate the synergistic effects of hydrogen sulfide (H_2S) and nitric oxide (NO) in enhancing salt stress tolerance in Cucurbitaceae seedlings (cucumber and bitter gourd), with emphasis on elucidating their roles in modulating growth performance, physiological responses, antioxidative defense systems, and cellular signaling mechanisms under salinity stress.

ii. The findings demonstrated that salinity stress significantly compromised seedling growth and metabolism, reducing fresh weight (FW), dry weight (DW), plant height (PH), chlorophyll content, nitrogen metabolism, and endogenous H_2S and NO levels, while increasing oxidative stress and suppressing antioxidative enzymatic activities.

iii. Exogenous application of NaHS (H_2S donor) and SNP (NO donor) effectively countered these detrimental effects by:

   a) Improving seedling growth, chlorophyll content, and nitrogen metabolism

   b) Restoring endogenous H_2S and NO levels

   c) Activating antioxidative defense mechanisms through enhanced activities of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (GPX), and glutathione S-transferases (GSTs)

   d) Reducing oxidative damage indicators such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and malondialdehyde (MDA)

iv. Inhibiting NO with cPTIO and L-NAME disrupted these protective mechanisms, highlighting NO's indispensable role in stress tolerance, while H_2S demonstrated partial compensatory effects under NO inhibition.

v. Simultaneous inhibition of both signaling molecules resulted in severe oxidative damage, compromised antioxidative defense, and impaired growth, confirming their synergistic and complementary interactions in stress mitigation pathways.

Societal Implications and Contribution to Sustainable Development Goals (SDGs)

i. Enhancing Agricultural Productivity in Saline Soils (SDG 2: Zero Hunger) Soil salinization poses a major threat to global food production. This study provides an effective strategy to improve salt tolerance in crops via the combined application of H_2S and NO, facilitating crop cultivation in saline-affected regions. By contributing to

sustainable intensification and improved yields in challenging environments, this research directly supports SDG 2: Zero Hunger, aiming to end hunger and ensure food security through sustainable agriculture.

ii. Promoting Sustainable and Environmentally Friendly Agricultural Practices (SDG 15: Life on Land) Conventional salinity management practices involve heavy use of chemical amendments, leading to long-term environmental degradation. This research advocates for the use of naturally occurring signaling molecules (H_2S and NO) to enhance stress tolerance, promoting eco-friendly, sustainable agricultural techniques. By protecting soil health, biodiversity, and ecosystem services, this aligns with SDG 15: Life on Land, which focuses on sustainably managing terrestrial ecosystems and combating land degradation.

iii. Economic Benefits for Farming Communities Improving crop resilience to salinity stress can reduce yield losses and stabilize farm incomes. For farmers, particularly in salinity-prone and marginal areas, this translates to economic benefits through better harvests, reduced dependency on costly soil remediation, and improved land-use efficiency, contributing to rural economic development and resilience.

In conclusion, this research not only advances scientific understanding of plant stress physiology but also proposes practical, sustainable, and scalable solutions to mitigate salinity-induced crop losses, thereby contributing meaningfully to global food security (SDG 2), sustainable land use and environmental protection (SDG 15), and farmer livelihoods, addressing some of the most pressing agricultural and societal challenges of our time.

Future Prospects

Despite significant advancements in understanding the roles of hydrogen sulfide (H_2S) and nitric oxide (NO) in salt stress tolerance, several areas remain open for further investigation. Future research should focus on elucidating the intricate molecular mechanisms underlying the synergistic interaction between H_2S and NO, particularly at the transcriptomic, proteomic, and metabolomic levels. Such studies will help identify key regulatory genes, signaling pathways, and protein modifications responsible for enhanced stress resilience.

Additionally, it is imperative to explore the crosstalk between H_2S, NO, and other phytohormones or reactive species (such as abscisic acid, jasmonic acid, salicylic acid, reactive oxygen and nitrogen species) to develop a comprehensive understanding of the integrated stress response network in plants. This knowledge could inform the design of targeted interventions to manipulate these pathways for improved crop performance under salinity stress.

Translating laboratory findings into practical applications requires the development of eco-friendly, cost-effective bio-stimulant formulations based on H_2S and NO donors. Seed priming or foliar application techniques could be optimized and tested across a wider range of economically important crops. Moreover, large-scale field trials under diverse agro-climatic and salinity conditions are essential to validate the effectiveness and scalability of these treatments.

Given that plants often face multiple simultaneous stresses in natural environments, future studies should also assess the potential of H_2S and NO in conferring tolerance to combined abiotic stresses, such as drought, heat, and heavy metal toxicity. Such multifaceted research will enhance our ability to develop resilient cropping systems in the face of climate change.

Ultimately, advancing our understanding and application of H_2S and NO signaling will contribute to climate-smart agricultural practices that promote sustainable productivity. This aligns with global efforts to achieve Sustainable Development Goals, especially SDG 2 (Zero Hunger) and SDG 15 (Life on Land), by improving food security and preserving ecosystem health in salt-affected regions.

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PUBLISHED RESEARCH/REVIEW ARTICLES

1. Kumari, R., Rakhra, G., Alsahli, A.A. *et al. Exploring the potential of signalling molecules hydrogen sulfide and nitric oxide in augmenting salt stress resilience in bitter gourd. BMC Plant Biol 25, 1008* (2025).
2. Kumari, R., Khan, M.N., Parrey, Z.A., Kapoor, P., Mir, B.A., Taziun, T., Parihar, P. and Rakhra, G., 2025. *Synergistic effects of hydrogen sulfide and nitric oxide in enhancing salt stress tolerance in cucumber seedlings. Physiologia Plantarum*, *177*(1), p.e70109.
3. Kumari, R., Kapoor, P., Mir, B.A., Singh, M., Parrey, Z.A., Rakhra, G., Parihar, P., Khan, M.N. and Rakhra, G., 2024. *Unlocking the versatility of nitric oxide in plants and insights into its molecular interplays under biotic and abiotic stress*. *Nitric Oxide*.
4. Mir, B.A., Kumari, R., Rakhra, G., Parihar, P., Singh, R., Raju, A.D., Srivastava, P.K., Prasad, S.M., Singh, R. and Gulliya, S., 2024. *Sulfur assimilation and regulation of abiotic stress via OMICS. Plant Stress, p.100630.*

BOOK CHAPTERS

1.) Taziun, T., Mir, B.A., Kumari, R., Akhtar, N. and Wani, A.K., 2024. Plant Metabolism and Abiotic Stress in Crops. *Plant Secondary Metabolites and Abiotic Stress*, pp.81-103.

2.) Mir, B.A., Taziun, T., Mir, M.R., Mir, T.U.G., Kumari, R., Wani, A.K. and Akhtar, N., 2024. Reactive Oxygen, Nitrogen, and Sulfur Species Under Abiotic Stress in Plants. Plant Secondary Metabolites and Abiotic Stress, pp.213-242.

3.) Mir, B.A., Kumari, R., Firdoos, A., Taziun, T., Aymen, U., Khan, M., Raju, A.D., Singh, R., Prasad, S.M., Singh, R. and Wani, S.H., 2024. Influence of Nanomaterials on Physiology and Antioxidant Defense Activities in Plants Under Abiotic Stress Conditions. In Innovative Methods in Horticultural Crop Improvement: Molecular Tools, Nanotechnology and Artificial Intelligence (pp. 117-149). Cham: Springer International Publishing.

4.) Kumari, R., Chattopadhyay, D., & Khurshid, N. (2024). Water management and irrigation innovations. In *Green Innovations: Modernizing Vegetable Cultivation* (pp. 43/61); [Radiant Flair Publication]. Print ISBN: 978-81-976308-5-9; E-ISBN: 978-81-976308-8-0.

CONFERENCES/WORKSHOPS

1. 6th International Conference Strategies and Challenges in Agricultural and Life Science for Food Security and Sustainable Environment (SCALFE - 2023) Venue: Himachal Pradesh University, Summer Hill, Shimla, HP, India APRIL 28 – 30, 2023. (Oral Presentation).
2. 4th International conference of Global Efforts on Agriculture Forestry, Environment and Food Security September 17-19, 2022, at Institute of Forestry, Tribhuvan University, Pokhara Campus Pokhara, Nepal (Oral presentation).
3. International Conference on Sustainability: Life on Earth 2021 (ICS-LOE 2021) held on 17-18 December at Lovely Professional University, Punjab (Poster presentation)
4. International symposium sustainable urban environment (ISSUE– 11-14 october,2022), at University of Petroleum and Energy Studies (UPES), Dehradun, India (Poster presentation).