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RESEARCH ARTICLE

Unravelling nitric oxide-hydrogen sulfide interplay in NaCl-mediated salinity resistance of moth bean [*Vigna aconitifolia* (Jacq.) Marechal]

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Abstract

Soil salinity causes oxidative stress, ion imbalance, osmotic anomaly, nutrient imbalance and changes in plant growth regulators. The study aims to investigate the salinity tolerance limit, involvement of nitric oxide (NO) and hydrogen sulfide (H_2S) and effects of different concentrations of exogenously supplied sodium chloride (NaCl) (50, 100, 150, 200 and 250 mM) on growth, physiological and biochemical parameters of the moth bean [*Vigna aconitifolia* (Jacq.) Marechal] seedlings. moth bean, a neglected and underutilised legume crop in India. The experimental results showed that salinity led to decrease in the plant growth [64.8% shoot length (SL), 58.6% root length (RL), 97.5% secondary roots], 19.3% fresh weight (FW), 16.01% dry weight (DW), (25.6%) water content, photosynthetic pigments (58.2% chl a, 20% chl b, 47.7% total chl and 63.9% carotenoids), 27.3% antioxidant contents and 23.7% increased malondialdehyde (MDA) content. The endogenous generation of NO (4.9%) and H_2S (58%) were reported under salinity stress compared to the control treatment. The osmolyte proline (184.2%) and enhanced activities of antioxidant enzymes, such as catalase (CAT) (25.22%), peroxidase (POD) (22.25%), superoxide dismutase (SOD) (17.63%) and polyphenol oxidase (PPO) (24.80%), up to 150 mM NaCl treatment, marking the tolerance limit of the moth bean. Principal component analysis (PCA) and correlation analysis revealed the antagonistic generation pattern of NO and H_2S, as well as their relationships with antioxidant enzymes under stress conditions. This is the first report on NO and H_2S generation and involvement in moth bean grown under salinity stress. The information obtained can be applied to augment salinity stress and enhance crop productivity.

Keywords: antioxidant enzymes; hydrogen sulfide; moth bean; nitric oxide; salinity

Introduction

Soil is vital for feeding the burgeoning global population through agricultural activities (1). Global agriculture is facing a significant challenge from soil salinity, which induces abiotic stress in crop plants, threatening crop productivity and ecological stability (2). Healthy soil is essential for realising most United Nations Sustainable Development Goals (SDGS) (3). Salinity affects an area of more than 1 billion hectares worldwide, in over 100 countries and is constantly increasing due to anthropogenic activities such as poor irrigation practices, climate change and industrial pollution (4, 5). High salinity causes the accumulation of ionic salts (Na^+, Cl^-) that become toxic and hamper water uptake, affecting the plant's functions and broadly altering nutrient cycling and ecosystem services (6). Severe soil salinity can lead to soil degradation and consequently, problems such as reduced agricultural productivity and compromised food security arise (7).

Moth bean (*Vigna aconitifolia* (Jacq.) Marechal), locally known as mat, matki, math, or mout bean, a drought-resistant, nutritionally rich legume pulse crop (subfamily Papilionoideae), is native to India and is grown in arid and semi-arid regions of South Asia (8). According to reports, the crop is cultivated in the arid, sandy tracts of India's driest state, Rajasthan. The crop is considered a neglected and underutilised crop (NUC) due to some of its antinutritional factors (ANF) (9). moth beans are increasingly recognised not merely as a supplementary pulse crop but as a promising candidate for sustainable agriculture as a source of food and fodder (10). Moth bean growth and physio-biochemical composition are influenced by salinity originating from irrigation or indiscriminate addition of fertiliser, pesticides, heavy metals, or industrial pollutants through surface runoff (9). Moth bean is considered a salinity-susceptible legume crop. Salinity affects crop growth by inducing phytotoxicity, which disrupts critical physiological processes such as nutrient uptake and photosynthesis and

impairs plant health, leading to a decline in the quality and yield of the moth bean crop (11).

Salinity stress is reported to involve an increase in endogenous H_2S, accompanied by an increase in NO (12). Analysis of previous reports indicates involvement of these gasotransmitters in various physiological and biochemical responses, such as seed germination (13), decrease in growth inhibition, photosynthetic characteristics and enhanced osmolyte contents (proline and glycine betaine), reduced oxidative stress that led to lowering of lipid peroxidation, electrolyte leakage and increased non-enzymatic antioxidants and activities of antioxidant enzymes as reported in common bean (12), soybean (14), alfalfa (15) and red kidney bean (16). This suggests their adaptive role through cross-talk involving H_2S-NO signalling and interactions during abiotic stress (17). Exogenous salinity stress was induced in the experimental study to evaluate the effects of salinity on growth and physio-biochemical parameters, as well as the involvement of gasotransmitters (NO and/or H_2S) in moth bean plants. The experiments aimed to gather information about the participation of these gasotransmitters in the moth bean, which has not been previously reported. This can further help develop strategies for augmenting salt stress tolerance in moth bean and contribute to sustainable water and soil management by devising better agricultural practices to mitigate the negative impacts of salinity stress.

Materials and Methods

Plant materials and treatments

Moth bean seeds (*Vigna aconitifolia* (Jacq.) Marechal); CV-RMO 25; purchased from Swami Keshwanand Rajasthan Agricultural University (SKRAU), Bikaner, Rajasthan, India). Moth bean (*Vigna aconitifolia* (Jacq.) Marechal) variety RMB-25 is an early-maturing variety. It takes approximately 62-70 days to help escape terminal drought. The variety shows an erect to semi-erect type with synchronised growth, maturity and high harvest index (>30%). The variety is also resistant to yellow mosaic virus (18). The seeds were stored in humidity-free containers for further experimental work. When used for experimentation, the seeds were sterilised with 1.0 % sodium hypochlorite (NaOCl) for 5 min and washed 5 times with distilled water (19). They were then soaked overnight at 25 °C in a plant growth chamber. The seeds were plated on transparent petri plates (8.0 cm diameter × 1.25 cm height) that were layered with distilled water-soaked Whatman paper. Each Petri plate contained twenty seeds. The experimental setup was constructed according to Table 1. Three biological replicates were kept for each treatment. Each Petri plate was treated with 1.0 mL of different NaCl concentrations and then watered daily with 500 µL of water. After seven days, when the full seedlings emerged, the moth bean seedlings were harvested to assess the different morphological, physiological and biochemical effects of control, moderate and severe salinity stress (Table 1).

Table 1. Experimental design

S.No.	Experimental Set-up	NaCl treatment (mM)
1.	1 (CK)	0
2.	2	50
3.	3	100
4.	4	150
5.	5	200
6.	6	250

CK: Control.

Growth/morphological parameters

Root-shoot lengths: After the experiments were terminated, ten seedlings from each Petri dish were randomly and thoroughly collected and washed with distilled water. The RL and SL of these seedlings were measured using a ruler. The shoot was measured from the shoot apical meristem to the root-shoot junction and RL was measured from the junction to the root tip. Additionally, the number of secondary roots was counted for each treatment by following the protocol described (20).

Fresh and dry weights: seedlings used for measuring the shoot-root lengths were dried using tissue paper to remove any external water content that adhered to the plant body after washing. Then, FW of three sets of 10 plants each were taken using an analytical balance. These three sets were dried at 60 °C for 48 hours in a hot oven. Dry weight (DW) was measured by an electronic weighing balance, following the protocol as described (20). FW and DW were used to determine and analyse the biomass and water content of the moth bean seedlings.

Water content: The water content was calculated following the standard protocol (21) and expressed as follows:

$$\text{Water content (g)} = \frac{\text{FW-DW}}{\text{FW}} \quad \text{(Eqn. 1)}$$

Stress tolerance index: The stress tolerance index (STI) is a useful method for determining the high-yield and stress tolerance potential of seeds, assessed for their ability to withstand salinity stress. Stress tolerance indices for root and shoot growth were estimated on the seventh day immediately after the experiment's termination by following a standardised protocol (22). The STI in the shoot (sSTI) and root (rSTI) were calculated using the following equations.

$$\text{sSTI (\%)} = \frac{\text{Shoot length of stress plant}}{\text{Shoot length of control plant}} \times 100 \quad \text{(Eqn. 2)}$$

$$\text{rSTI (\%)} = \frac{\text{Root length of stress plant}}{\text{Root length of control plant}} \times 100 \quad \text{(Eqn. 3)}$$

Physio-biochemical analysis

Control and NaCl-treated seedlings were used to determine the following physio-biochemical parameters that influence the growth of the moth bean crop.

Analysis of photosynthetic pigments

Photosynthetic parameters, such as chlorophyll a, b, total chlorophyll and carotenoids, were determined by taking fresh leaf samples (0.01 g) from control and treated plant seedlings by the Dimethyl sulfoxide (DMSO) method (23). The colour absorbance was recorded at 645 nm, 663 nm and 480 nm by a UV-Vis spectrophotometer. The concentrations of chlorophyll a, chlorophyll b, total chlorophyll and carotenoid were calculated (24), using the following equation:

$$\text{Chlorophyll a: } 12.7(A663) - 2.69(A645) \times \frac{V}{1000} \times W$$

(Eqn. 4)

$$\text{Chlorophyll b: } 12.9(A645) - 4.68(A663) \times \frac{V}{1000} \times W$$

(Eqn. 5)

$$\text{Total Chlorophyll: } 20.2(A645) + 8.02(A663) \times \frac{V}{1000} \times W$$

(Eqn. 6)

$$\text{Carotenoid: } A480 + (0.114(A663) - (0.638 - A645) \times \frac{V}{1000} \times W$$

(Eqn. 7)

Where "W" is the fresh weight of the leaves, "V" is the extraction volume and it is expressed in terms of mg g^{-1} fresh weight.

Estimation of lipid peroxidation: Oxidative stress induced by salinity causes lipid peroxidation, which was determined in the moth bean seedlings (0.3 g) by measuring the MDA content formed by the thiobarbituric acid (TBA) reaction, according to the protocol described (25).

Quantification of Proline: Proline is a cellular osmolyte and is considered a nonenzymatic antioxidant that imparts tolerance against salinity stress (26). Proline was determined in moth bean seedlings (0.3 g) using the protocol described (27). Absorption was measured at 520 nm using a UV-Vis spectrophotometer. The proline concentration was determined based on a standard curve and expressed as µmol g^{-1} DW.

Determination of Antioxidant Activity: The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was used to quantify the antioxidant activity of the whole plant extracts from the moth bean seedlings (0.1 g), following the given protocol (28). The scavenging ability of DPPH (% inhibition) was carried out in triplicate using the following equation:

$$\text{DPPH scavenging effect (\% of inhibition)} = \frac{(A0 - A1)}{A0} \times 100$$

(Eqn. 8)

In this equation, A_0 is the absorbance value of the control and A_1 is the absorbance value of the plant extracts. The inhibition percentage was determined against a NaCl concentration treatment (mM).

Enzyme extraction: Healthy and whole plant seedlings (0.4 g) were randomly collected from Petri dishes treated with NaCl and the control and then well-grounded with the help of a cold pestle and mortar in 4 mL of 50 mM potassium phosphate buffer (pH 7.6) containing EDTA (0.5 mM). The homogenised plant tissues were centrifuged (Remi India) at 10000×g for 20 min at 4 °C. The supernatant was collected in well-labelled Eppendorf tubes, preserved at -20 °C and further used to determine biochemical parameters, such as antioxidants and H_2S synthesising enzymes assays, on a protein basis. The Bradford protocol quantified soluble proteins (29). Determination of antioxidant enzymes such as CAT activity (30), Guaiacol peroxidase (POD) (31) and SOD (32) was performed according to the respective protocols.

Estimation of catalase activity: Catalase (CAT; EC 1.11.1.6) enzyme activity was measured by calculating the consumption of H_2O_2 according to the protocol in both treated and control seedlings. All the measurements were carried out by using an enzyme extract (50 µL) in triplicate. The dissociation of hydrogen peroxide (H2O2) was measured using the extinction coefficient of 39.4 M^{-1} cm^{-1}. One unit (U) of CAT activity is the sum of enzyme dissociation, 1.0 µmol H_2O_2 min^{-1}.

Estimation of guaiacol peroxidase (POD) activity: Guaiacol peroxidase (POD, EC 1.11.1.7) is a category of peroxidases that acts as an antioxidant system in H_2O_2 elimination. The enzyme, a key component in lignin biosynthesis, catalyses the oxidation of many phenolic compounds at the expense of H_2O_2. The enzyme extract (50 µL) was used for measurement of the POD enzyme activity. It was based on the determination of guaiacol oxidation (extinction coefficient 26.6 mM L^{-1} cm^{-1}) at 470 nm by H_2O_2. All the measurements were carried out in triplicate.

Estimation of superoxide dismutase activity: Superoxide dismutase (SOD, EC 1.15.1.1) is a group of enzymes that constitute a crucial component of the antioxidant defence in plant systems, as it plays a first-line defence during oxidative stress. The enzymatic action of SOD is to catalyse the disproportionation of O_2^- to H_2O_2 and O_2 (33). The enzyme extract (100 µL) was used for measurement of the SOD activity. All the measurements were carried out in triplicate.

Estimation of polyphenol oxidase activity: Polyphenol oxidase activity was determined using a standardised protocol (34). The enzyme extract (100 µL) was used for measurement of the POD enzyme activity. All the measurements were carried out in triplicate.

Measurement of endogenous H_2S content: The enzyme L-cysteine desulfhydrase (L-CD_S; EC 4.4.1.28) activity was measured in triplicate using a modified methylene blue protocol to assess the endogenous H_2S generation in the seedlings (0.4 g) (35). One unit of enzyme activity for L-CD_S is defined as the amount of enzyme that generates 1 µmol min^{-1} H_2S under the stated assay conditions and is expressed as units per milligram (U mg^{-1}).

Measurement of endogenous NO content: Nitric oxide content was determined from whole plant seedlings (0.4 g) of moth bean as per a standardised protocol (36). The determination of NO content was done by comparing it to a sodium nitrite $NaNO_2$ standard curve.

Statistical analysis

The data were expressed as the mean ± standard error (M ± SE), statistical analyses and One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test were performed using GraphPad Prism version 10.4.2 for Windows using GraphPad Software, Boston, Massachusetts, USA, with $p < 0.05$ considered significant.

Results and Discussion

Plant growth and biomass parameters: NaCl stimulates at lower doses, but is harmful at higher doses

Moth bean plant seedlings showed a dose-dependent negative influence on NaCl treatment, except for the 50 mM treated group, which exhibited significant stimulatory growth in SL

(5%) and RL (32.8%) compared to the control. The maximum reductions in RL (58.6%) and SL (64.8%) were observed in the 250 mM treatment compared to the control. Similarly, secondary roots were not significantly affected at 50 mM but declined sharply as NaCl concentrations increased 40.98% (100 mM) and almost disappeared in 96.7% (200 mM) and 97.54% (250 mM) NaCl solutions, clearly indicating the toxicity of NaCl and the plant's tolerance limit (Fig. 1, 2a, 2b, 2c).

Similarly, exogenous NaCl treatment influenced the FW and DW of seedlings, with a notable decrease at a 250 mM concentration compared to the control. The seedlings showed an increase of 11.41% FW in the 50 mM NaCl treatment, but afterwards, a decline occurred. Dry weight showed a dose-dependent decrease, except the 50 mM treated group exhibited the least decrease (4.50%) compared to the other groups (Fig. 3a, b).

However, the water content in control and NaCl-treated seedlings showed that the initial treatments had significant positive responses, with increases of 21.19% and 18.76% in 50 mM and 100 mM NaCl treatments, respectively, compared to the control. However, the water content remained insignificant in the 150 and 200 mM NaCl treatments but significantly declined in the highest dose of NaCl treatment (250 mM) compared to control seedlings (Fig. 3c).

The stress tolerance index (STI), a helpful method for determining the high yield and stress tolerance potential of seeds and seedlings, was also assessed for salinity stress in the moth bean. The results for the root and shoot parameters showed differential behaviour with NaCl treatment. The highest dose of NaCl treatment (250 mM) showed a decrease in root STI (rSTI) by 55.2% and in shoot STI (sSTI) by 59.5%. The index results for sSTI and rSTI, both showed positive values at 50 mM, with 56% and 11%, respectively. However, NaCl treatment at 100 mM showed an insignificant change in the case of rSTI compared to the control and 50 mM treatment, but not in the case of sSTI, suggesting that shoots are less affected than roots (Fig. 4a, b).

Since it is well known that salinity, in general, negatively affects crop plants, depending on the tolerance limits (37) determined by the cumulative responses of both enzymatic and nonenzymatic antioxidant activities to counteract oxidative stress and toxicity induced by salinity stress (38). As results showed, suppressed plant growth, evidenced by a decrease in root-shoot lengths, secondary roots and biomass (both FW and DW) of the moth bean seedlings in a dose-dependent manner. The concentration of NaCl above 100 mM led to the plant cell shrinking, dehydration and osmotic changes, affecting the plant's decreased capability to absorb water and nutrients, such as phosphate. Interestingly, low doses of NaCl (such as 50 mM and 100 mM) were found to be stimulatory for the moth bean plant growth, as visible in the morphological features (Fig. 1). This could be attributed to improved nutrient assimilation. The secondary roots are known to absorb the nutrients, such as phosphate, required for growth and development (37). Salinity caused a sharp decline in the number of secondary roots above 100 mM NaCl, which may be due to interference (osmotic imbalance) in the absorption of vital nutrients, affecting plant cell division and growth. Similar responses are observed in other legumes, such as soybeans (39) and peas (40). This reduction can also be correlated with decreased leaf area, reduced pigment synthesis and membrane damage resulting from the diversion of resources for the synthesis of osmolytes or other antioxidants.

Physiological and biochemical parameters: NaCl modulates physiological and biochemical responses

Salinity stress exhibited a general decline in photosynthetic pigments (chlorophyll a, chlorophyll b, total chlorophyll (a + b) and carotenoids) compared to the control in moth bean. Chlorophyll a significantly declined, with 58.2% and 42.9% recorded in 200 mM and 250 mM NaCl treatments, respectively. In contrast, Chlorophyll b showed a significant increase of 9.09% and 17.2% in 50 mM and 100 mM NaCl treatments, respectively, but declined at higher concentrations. Total Chlorophyll and carotenoid content followed the same trend as chlorophyll a (Fig. 5a, b, c, d). Lipid peroxidation occurs due to increased levels of free radicals and H_2O_2 in oxidative stress (41). The increased level of MDA indicates the degree of membrane damage (42). The concentration of MDA gradually increased

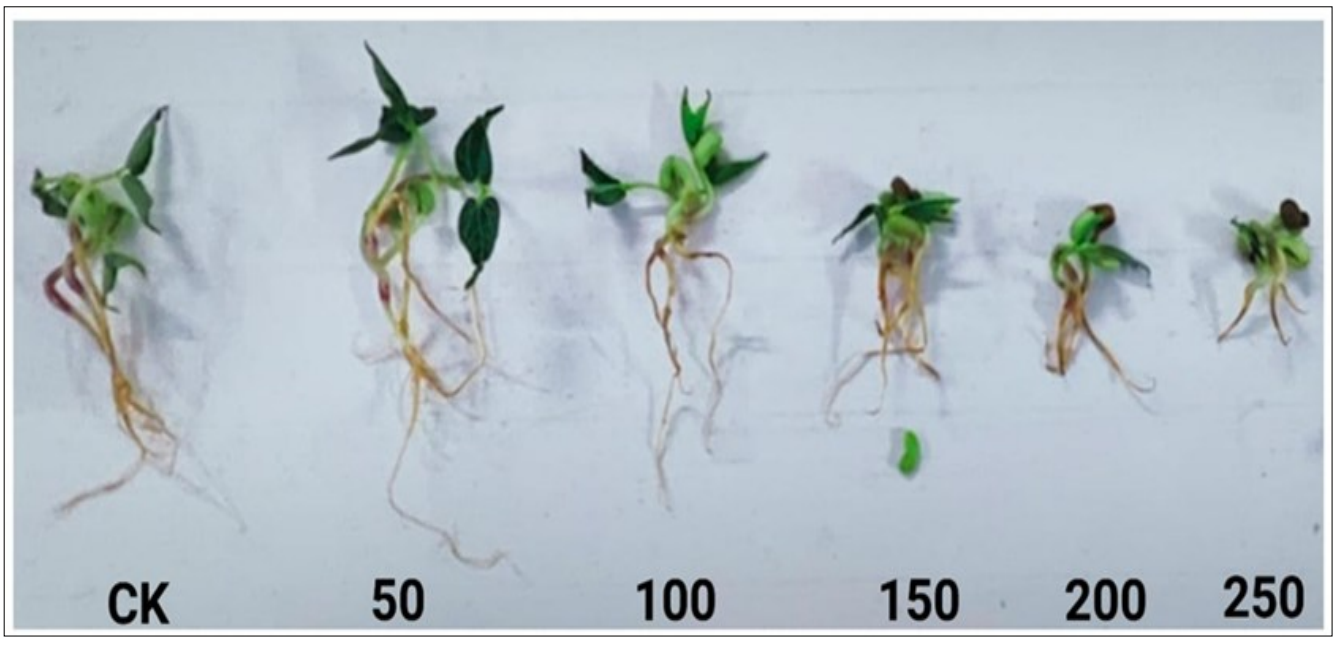

Fig. 1. The morphology of seven-day-old Moth bean seedlings treated with different NaCl concentrations.

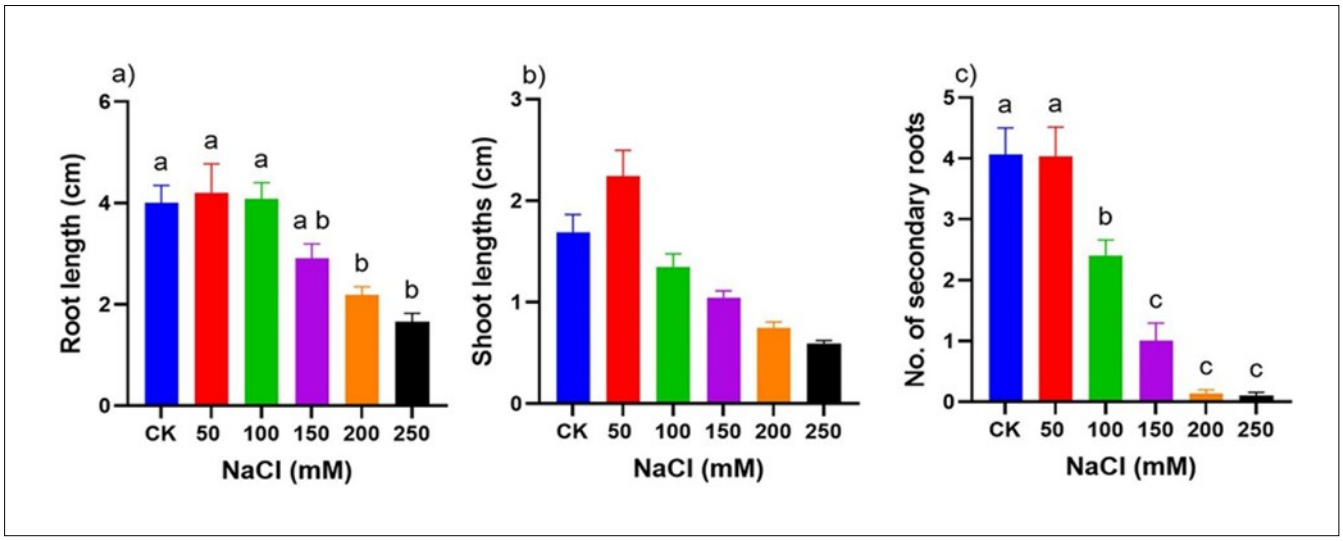

Fig. 2. Effects of exogenous NaCl treatments on seven-day-old moth bean (*Vigna aconitifolia*): (a) root lengths, (b) shoot length, (c) number of secondary roots. Values are ± mean standard error (MSE) and letters indicate significant mean differences at the $p < 0.05$ level (one-way ANOVA), analysed using the Tukey test.

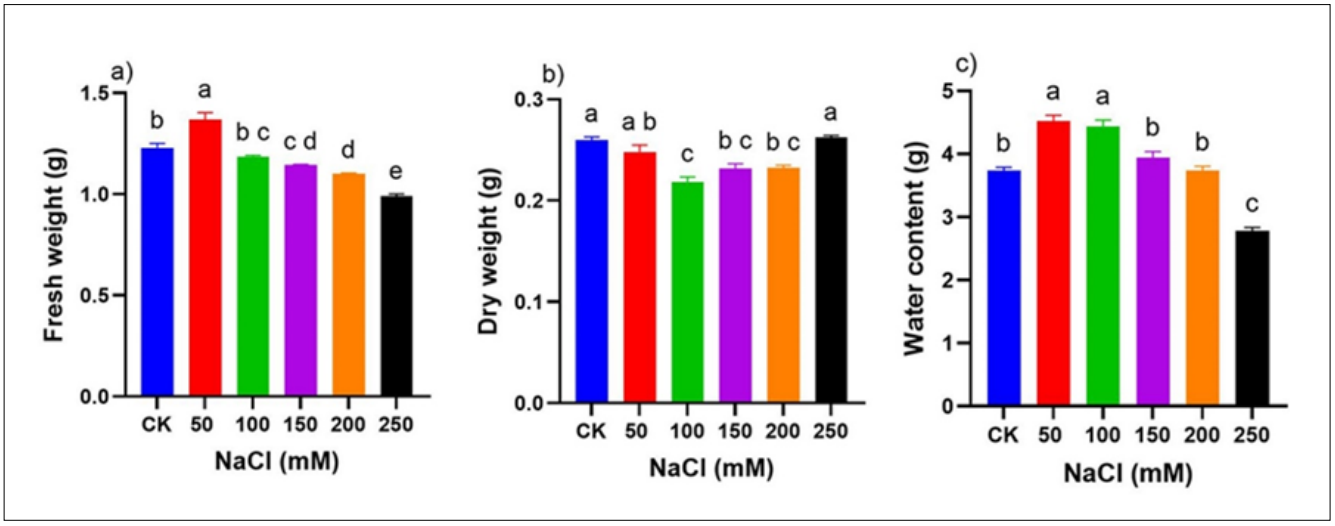

Fig. 3. Effects of NaCl treatments on seven-day-old moth bean (*Vigna aconitifolia*) seedlings. (a) fresh weight, (b) dry weights, (c) water content. Values are ± mean standard error (MSE) and letters indicate significant mean differences at the $p < 0.05$ level (one-way ANOVA), analysed using the Tukey test.

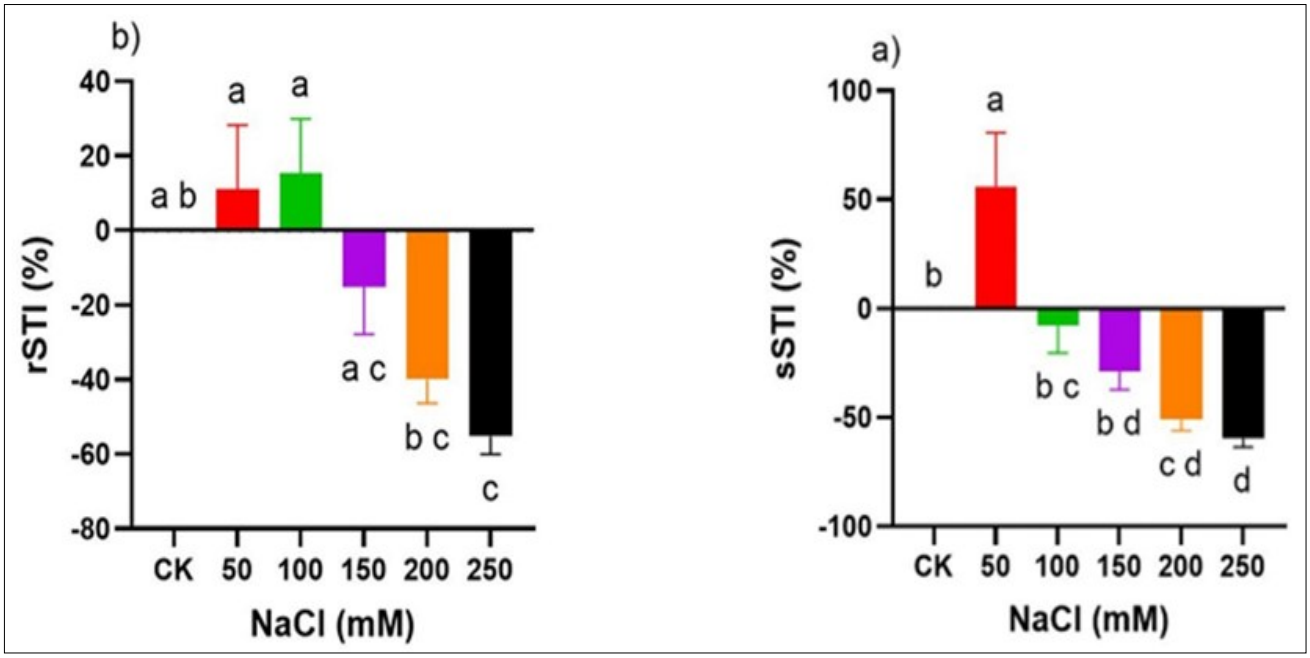

Fig. 4. Effect of exogenous NaCl on root and shoot stress tolerance index of seven-day-old moth bean (*Vigna aconitifolia*) seedlings. (a) Root salt tolerance index, (b) Shoot salt tolerance index. Values are ± mean standard error (MSE) and different letters indicate significant mean differences at the $p < 0.05$ level (one-way ANOVA), analysed using the Tukey test.

and reached a maximum value of 23.7 % at 200 mM, compared with the control, then declined (Fig. 6a). However, exogenous NaCl application significantly enhanced the accumulation of proline in seven-day-old moth bean seedlings compared to the control. The proline content gradually increased with NaCl treatment, reaching a maximum at 200 mM NaCl, nearly double (184.2 %) the content compared to the control (Fig. 6b). The antioxidant activity of the moth bean extracts was determined by DPPH assay. Under salt stress, the antioxidant activity was negatively correlated, following a dose-dependent response. The results showed that the CK had the highest antioxidant activity level in the DPPH assay, with a value of 47.92%. The data showed that lower NaCl treatments (50 and 100 mM) have a less significant impact on the antioxidant, with values of 42.8% and 43.1% (Fig. 6c).

The increase in oxidative stress can be correlated with our experimental finding of a dose-dependent increase in MDA content compared to the control. In contrast, the photosynthetic pigments, especially chlorophyll a, total chlorophyll and carotenoids, show a negative correlation. However, chlorophyll b exhibits an increased level until 150 mM salinity levels but declines at higher concentrations due to ion toxicity or damage to the photosynthetic machinery caused by oxidative stress. The results of the experiments clearly connect the salinity-triggered oxidative damage as seen in proteins, enzymes and lipids (43).

Further, the increased lipid peroxidation-based membrane damage and decreased photosynthetic pigment content negatively affect plant growth and development, leading to a decline in overall growth and development. In response, plants produce counteractive measures, such as non -enzymatic antioxidants, phenolics, carotenoids, proline and ascorbic acid, which scavenge ROS and mitigate damage (38) as reported by our finding of the synthesis of osmolyte proline content up to 250 mM NaCl concentration. If plants are removed from salinity stress, they regain their potential (data not shown). The imposition of exogenous NaCl treatment in the moth bean seedling caused a decline in total soluble protein (TSP) content at low NaCl treatment, but an increase (2.74%) was observed at 200 mM compared to the control (Fig. 7a). These soluble proteins also play a key role in osmoregulation.

Enzymatic antioxidant activities are upregulated to the tolerance limit

Antioxidant enzymes, such as CAT, POD, SOD and PPO, impart enhanced tolerance to salinity stress by counteracting oxidative stress (44). The defence begins by augmenting the first-line shield through increased activity of the SOD enzyme. It converts the superoxide radicals (O_2^-) into less harmful H_2O_2 molecules. After that, the second line of defence starts by converting H_2O_2 into H_2O and O_2 with the help of CAT and POD enzymes, mitigating oxidative stress (45). Another line of

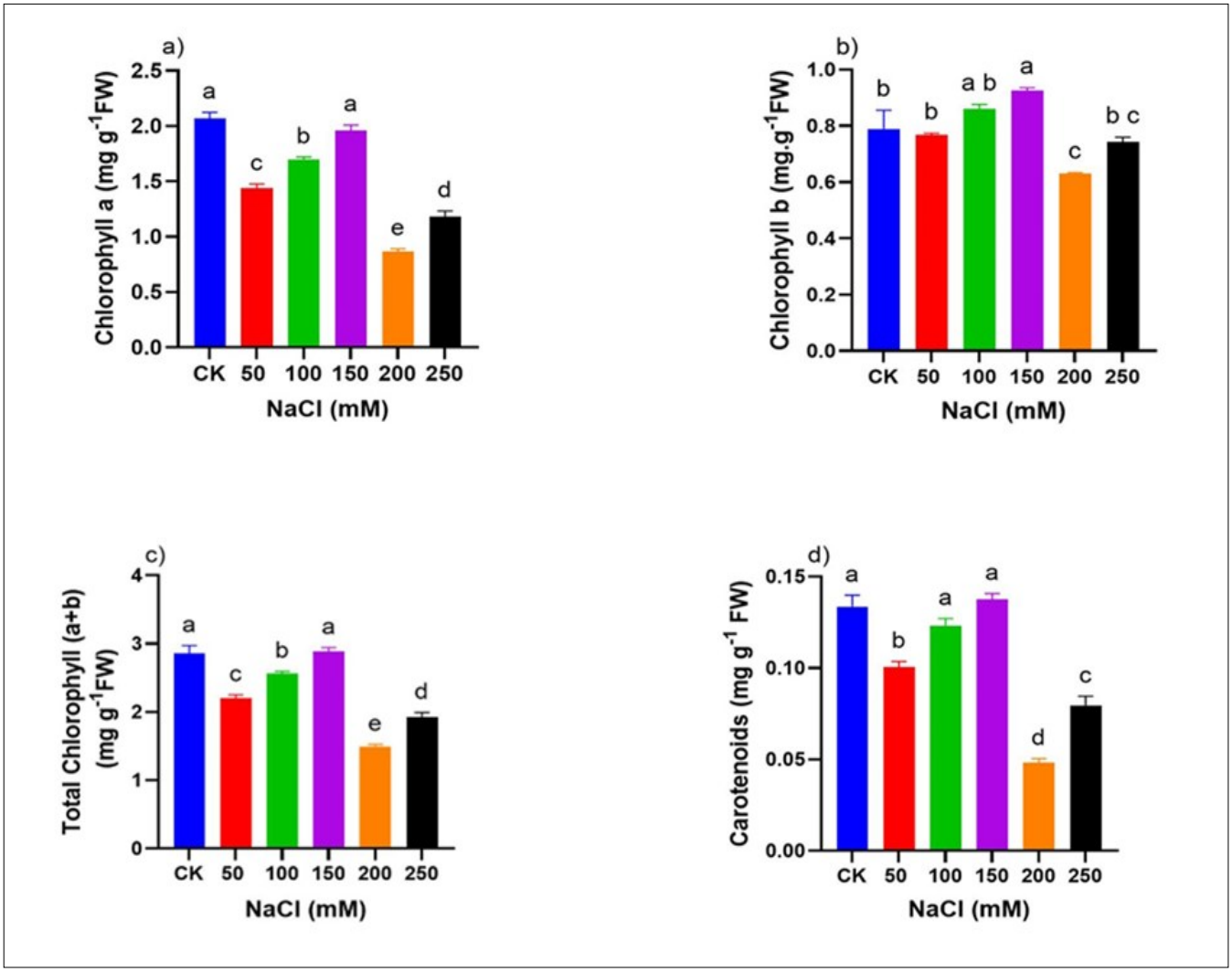

Fig. 5. Effect of exogenous NaCl treatments on the photosynthetic pigments of seven-day-old moth bean (*Vigna aconitifolia*) seedlings. (a) Chlorophyll a, (b) Chlorophyll b, (c) Total chlorophyll, (d) Carotenoids. Values are ± mean standard error (MSE) and letters indicate significant mean differences at the $p < 0.05$ level (one-way ANOVA), analysed using the Tukey test.

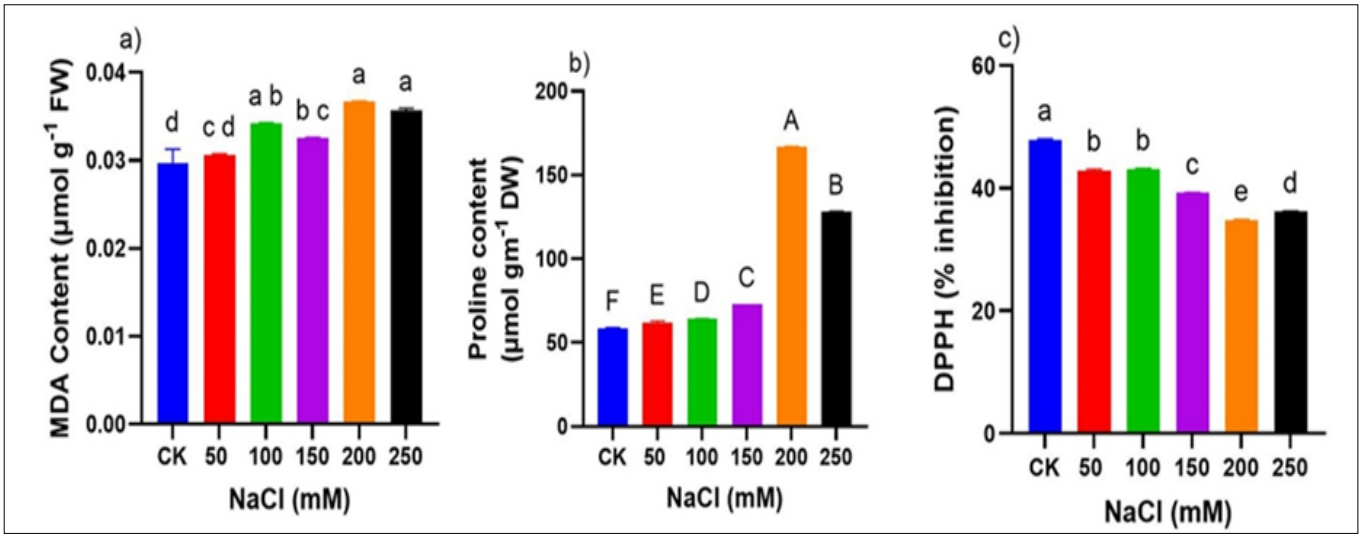

Fig. 6. Effect of exogenous NaCl treatments on seven-day-old moth bean (*Vigna aconitifolia*) seedlings. (a) MDA content, (b) Proline content, (c) DPPH% inhibition. Values are ± mean standard error (MSE) and letters indicate significant mean differences at the $p < 0.05$ level (one-way ANOVA), analysed using the Tukey test.

defence against stress is taken up by PPOs. These are a group of Cu-containing enzymes, involved in the catalysis of the oxidation of several phenols to *o*-quinones. These products, being highly reactive, undergo non-enzymatic secondary reactions and form brown complex polymers and cross-linked polymers with protein functional groups that help make the outer layer defensive (46). The salinity imposed by NaCl in the experiment clearly showed activation of the antioxidant system at low and moderate levels, up to 150 mM, compared to the control. There was a significant upregulation of 25.2%, 22.2% and 17.5% in CAT, POD and SOD activity, respectively, at 150 mM NaCl treatment compared to the control. After this level, the enzyme activities declined progressively (Fig. 7b, c, d). On the other hand, Moth bean seedlings treated with NaCl followed the same pattern in PPO enzyme activity as reported for CAT, POD and SOD. The enzyme exhibited an increase in activity of 24.8% at setup with 150 mM NaCl, followed by a decline compared to the control (Fig. 7e).

These results indicate that moth bean seedlings treated with NaCl enhanced antioxidant enzyme activities up to 150 mM NaCl treatment, by synthesis protective machinery such as proline and caused upregulated antioxidant enzymes (CAT, POD, SOD and PPO synthesis but declined thereafter, indicating the optimum salinity tolerance limit (150 mM) in moth bean that help preserving cellular function. It is important to note that the upregulation is almost equal in all four enzymes, suggesting the upper limit of the Moth bean's tolerance to salinity stress.

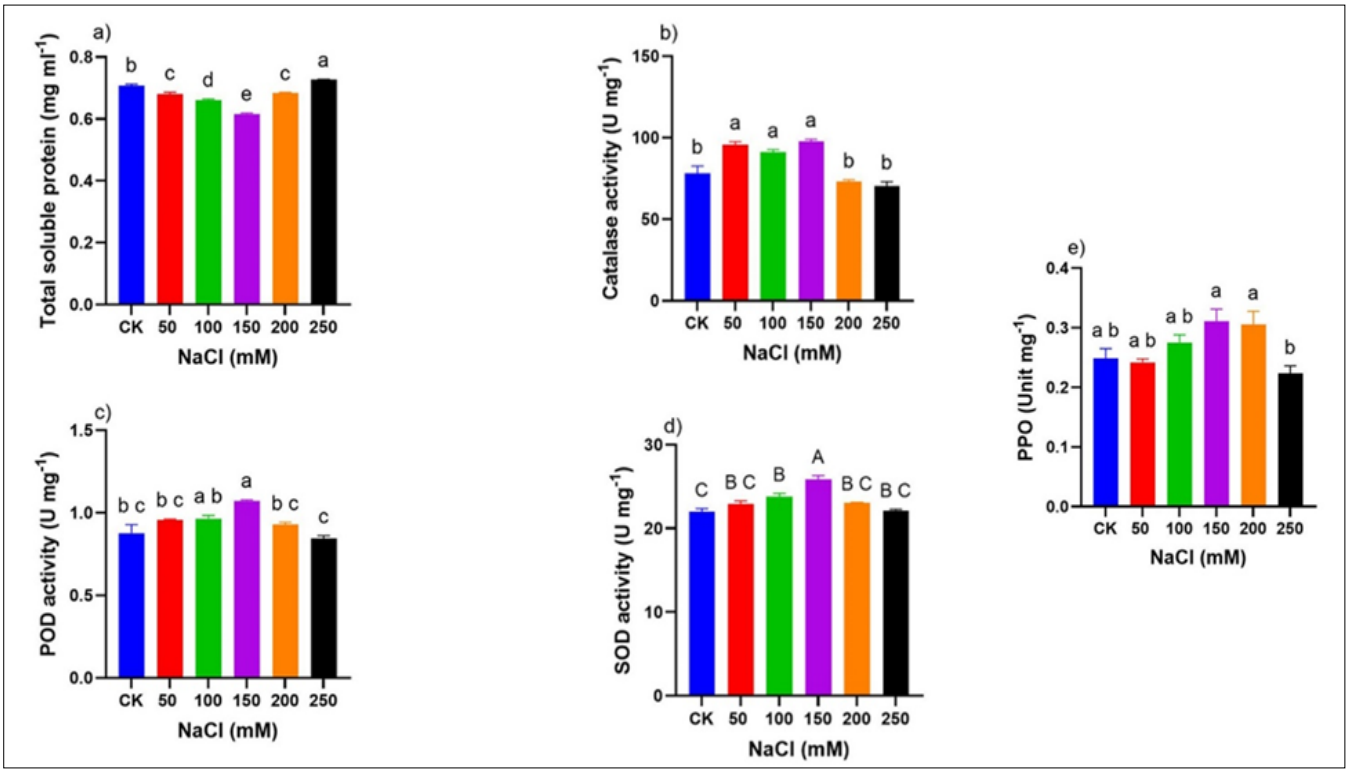

Fig. 7. Effect of exogenous NaCl treatments on total protein contents and antioxidant enzymes in seven-day-old moth bean (*Vigna aconitifolia*) seedlings. (a) Total soluble proteins, (b) Catalase (CAT), (c) Guaiacol peroxidase (POD), (d) Superoxide dismutase (SOD), (e) Polyphenol oxidase (PPO). Values are ± mean standard error (MSE) and letters indicate significant mean differences at the $p < 0.05$ level (one-way ANOVA), analysed using the Tukey test.

Determination of gasotransmitters (H_2S and NO): Antagonistic modulation during salinity stress

Salinity stress induced by NaCl in the moth bean seedlings influenced H_2S synthesising enzyme (L-CDs) activity. The observed endogenous enhancement was 58% and 51% H_2S generation in 200- and 250-mM treatments, respectively, compared to the control. Similarly, NaCl treatment showed a stimulatory role on NO generation that remained significant till 200 mM treatment (4.9%) and afterwards declined marginally compared to the control (Fig. 8a, 8b, 9).

Under salinity stress, a synchronised increase in H_2S and NO is reported in legumes (14), common beans (12) and alfalfa (*Medicago sativa*) (13), imparting salinity stress tolerance. There are several enzymes reported to be involved in the synthesis of H_2S, including l-/d-ysteine desulfhydrase (l-/d-DES), sulfite reductase (SiR) and cyanoalanine synthase (CAS), which utilise cysteine as a substrate (47). Another gasotransmitter, NO, is synthesised from diverse sources of substrate and enzymes and can even be produced by non-enzymatic sources in both plants and animals (48), making it more flexible in generation and action.

These two gasotransmitters show crosstalk and interconnection with themselves (37) and have common connections with other signalling molecules, such as ethylene and polyamine (49), phytohormones to ensure cellular homeostasis against stress conditions (50). These gasotransmitters are well reported to be endogenously induced, inducing the synthesis of photosynthetic pigments, regulating carbohydrate metabolism, stimulating proline synthesis and controlling the gene expression of antioxidant enzymes (17).

The salinity response shown by moth bean through the generation of gasotransmitters (NO and H_2S) is notably unique, with a trend in antioxidant enzyme activity. Nitric oxide showed an increasing and synchronised trend with increasing antioxidant enzymatic activities. On the other hand, the H_2S level was inversely related, as low levels of H_2S were associated with upregulated antioxidant enzyme activity, while high levels were associated with low antioxidant enzyme activity. The experimental evidence suggested that both gasotransmitters are involved in regulating antioxidant activities and salinity-induced responses in the moth bean. Such involvement of NO is reported in soybean (14), red kidney bean (16), H_2S in alfalfa (15) and both NO and H_2S in common bean (12).

Correlation analysis, heat map and principal component analysis: Diving deep into relationships

Pearson's correlation coefficients (r) were calculated based on the mean values of antioxidant enzymes (CAT, POD, SOD and PPO) and gasotransmitters (NO and H_2S) traits to measure the association under control and stress conditions. The impact of salinity is presented in a heat map in Fig. 10a. Under experimental conditions, a moderate negative correlation ($r = 0.53$, $p < 0.05$) exists between H_2S and NO, indicating their opposite responses during salinity stress in the moth bean. Gasotransmitter individually but differentially influenced antioxidant enzymes. In general, NO showed a positive correlation with all antioxidant enzymes, whereas H_2S showed either a very weak or negative correlation. Nitric oxide showed a weak positive correlation with CAT ($r = 0.33$) and PPO ($r = 0.35$) but a strong positive correlation with POD ($r = 0.76$, $p < 0.05$) and SOD ($r = 0.75$, $p < 0.05$). However, H_2S showed a very weak correlation with CAT ($r = 0.06$) and PPO ($r = 0.10$), as well as a very weak or negative correlation with POD ($r = 0.18$) and SOD ($r = 0.05$).

The PCA analysis of the biochemical traits, antioxidant enzymes and gasotransmitters (H_2S and NO) in moth bean are shown in Fig. 10b. The first two components (PC1 and PC2) obtained by the PCA accounted for 73.28% of the variability. PC1 accounted for 47.46% of the variability and divided MDA, chl a, total chl and carotenoids into negative values, while the other phytochemical parameters, such as proline and H_2S, showed positive values. PC2 accounted for 25.83% of the variability. It divided TSP, chl b, NO, POD and SOD into positive values and the other phytochemical parameters, such as PPO and CAT, into negative values. Longer vectors indicate that the variable has a strong influence on the corresponding component(s) and therefore contributes significantly to the dataset's variability. The biplot shows that NO and H_2S are generated but exhibit different behaviours under salinity stress. The effect of exogenous NaCl treatment on the moth bean seedling, in the form of salinity, affected various plant growth and development parameters.

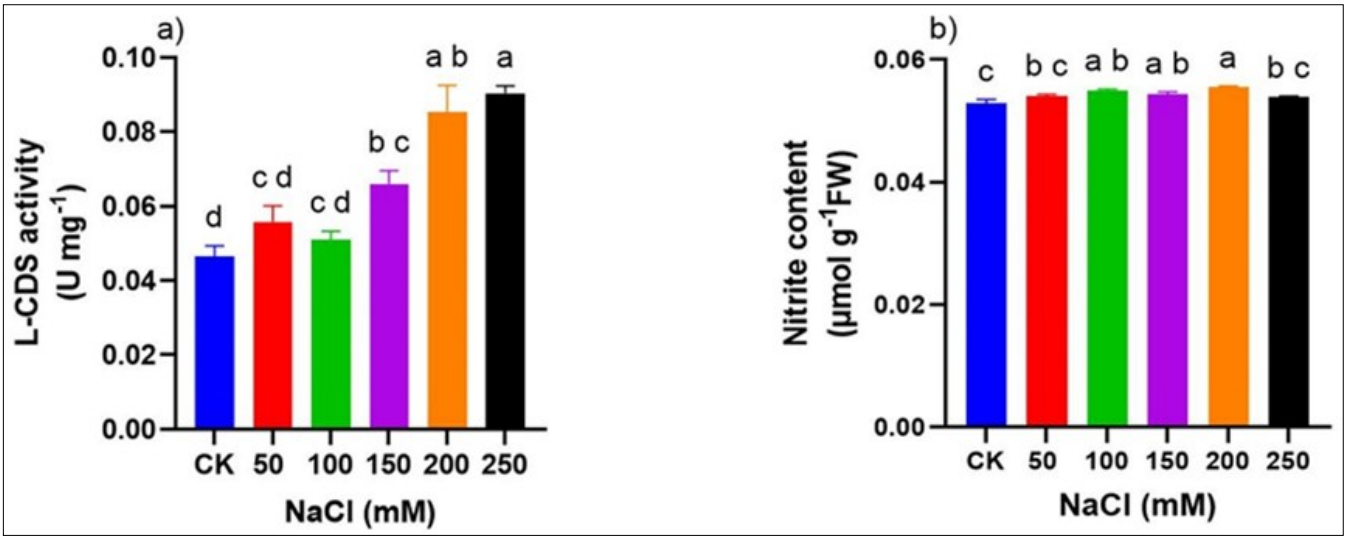

Fig. 8. Effect of exogenous NaCl treatments on the gasotransmitters synthesis in seven-day-old moth bean (*Vigna aconitifolia*) seedlings. (a) Nitric oxide, (b) H_2S synthesis. Values are ± mean standard error (MSE) and letters indicate significant mean differences at the $p < 0.05$ level (one-way ANOVA), analysed using the Tukey test.

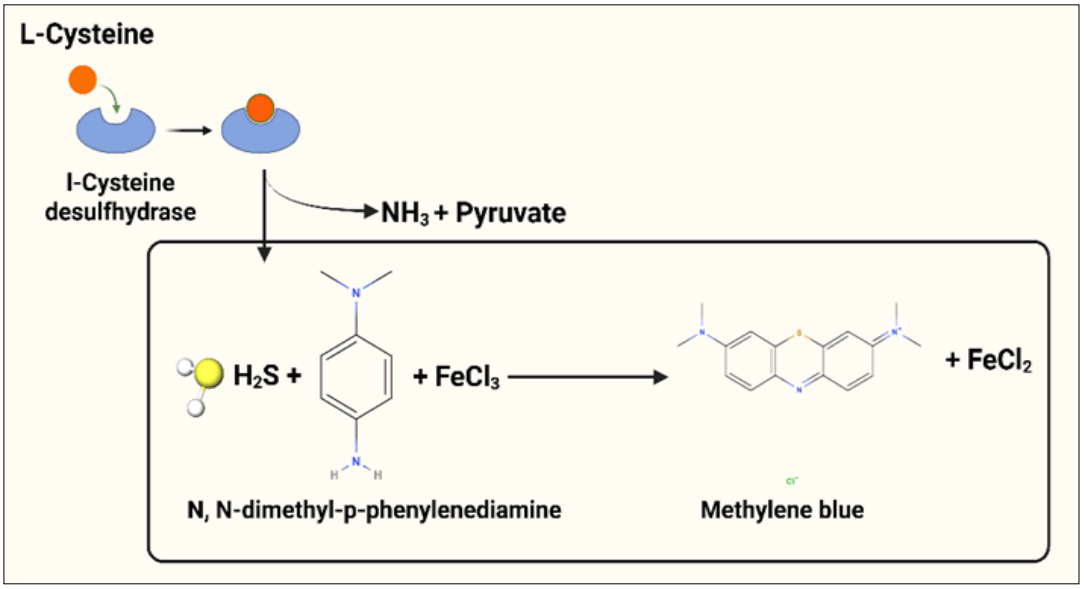

Fig. 9. Diagrammatic representation of the mechanism of synthesis and determination of H_2S in the plant system.

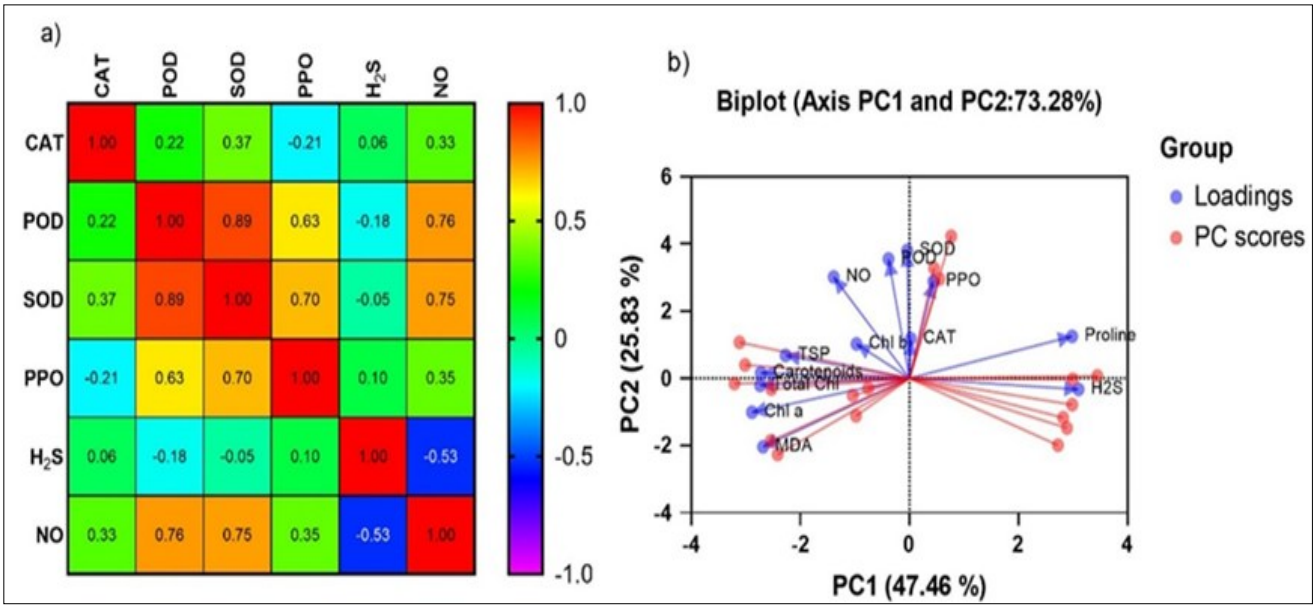

Fig. 10. (a) Heat maps of Correlation analysis (Pearson's correlation) between antioxidant enzymes and gasotransmitters (NO and H_2S). (b) A two-dimensional principal component analysis (2D-PCA) illustrates the relationship between physio-biochemical properties, antioxidant enzymes and gasotransmitters under salinity stress.

Gasotransmitters, including H_2S and NO synthesis and interaction, have not yet been reported in moth beans to date, which warranted investigation into their synthesis, trends, patterns and involvement in moth beans during salinity stress. The encouraging results revealed their endogenous synthesis and involvement under salinity stress with different doses of NaCl treatment, suggesting an integrated role in signalling and regulation. The enzyme and protective proline biomolecules showed positive reactions with enhanced NO and low levels of H_2S, as reflected by correlation analysis and PCA results. Overall, it can be stated that H_2S and NO are involved in the regulation of salinity stress induced by NaCl and further research is required to gain a deeper understanding.

Conclusion

The experimental work demonstrated the involvement and generation of endogenous NO and H_2S, as well as the synthesis of osmolytes and the upregulation of antioxidant enzymatic activities (CAT, POD, SOD and PPO) at concentrations up to 150 mM NaCl treatment given to the moth bean. Seeds treated with NaCl exhibited dose-dependent effects, negatively affecting growth as indicated by reductions in morphological parameters, including shoot and root lengths, as well as the number of secondary roots. The lower concentration of NaCl (up to 100 mM) showed lesser effects and was even found to be stimulatory; however, higher concentrations led to an extreme reduction, indicating NaCl toxicity. Stress tolerance indices showed differential responses in sSTI and rSTI. sSTI was good up to moderate NaCl treatments but declined in higher concentrations. The analysis of physiological and biochemical aspects revealed a general decline in photosynthetic pigments, antioxidant levels (as determined by the DPPH assay) and an increase in oxidative stress, as indicated by enhanced MDA content. However, contrary to this, the proline content and activation of antioxidant enzyme systems remain unchanged up to a 150 mM NaCl treatment, suggesting that beyond this treatment level, enzymatic activities are inhibited due to disturbances in ion homeostasis and osmotic balance, leading to a failure in imparting salinity tolerance. The gasotransmitter NO content increased with increasing NaCl

doses, opposite to H_2S synthesis. As the literature review revealed that there is no work available on the moth bean regarding gasotransmitters, the results of these experiments unveiled the generation and involvement during salinity stress. Further work needs to be done to fill the gaps to increase the understanding of physiology, signalling mechanisms and crosstalk under abiotic stresses pertaining to these gasotransmitters.

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Authors' contributions

KG and YPS carried out experimental work, data analysis, validation and contributed to the writing of the manuscript. AKM was responsible for conceptualisation, supervision, experimental work, result analysis, verification, manuscript writing, editing and overall review. KS contributed to the conceptualisation, supervision and critical review of the manuscript. S and BK were involved in the experimental work and participated in the manuscript review. DS handled the sectional composition, thoroughly reviewed, edited and final quality checked the manuscript. All authors have read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring its integrity and accuracy.

Compliance with ethical standards

Conflict of interest: The authors declare no conflicts of interest.

Ethical issues: None

Declaration of generative AI and AI-assisted technologies in the writing process: During the preparation of this work, the author(s) used Grammarly Premium for the betterment of the language of the manuscript. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

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