Ammonium nitrate enhances salinity stress tolerance by inducing physiological and phytohormonal regulation changes in *Vigna radiata* L.

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Highlights

- Soil salinity reduced growth and ultimately diminished the final productivity
- Salinity stress reduced aboveground fresh and dry biomass and chlorophyll contents
- Salinity stress increased H_2O_2, MDA, organic solutes, and antioxidant enzymes
- Nitrogen supplementation reduced H_2O_2 and MDA but improved other parameters
- Ammonium nitrate improved salinity tolerance and mitigating damage due to salinity

Abstract

Mungbean is a key leguminous crop species highly regarded for its nutritious properties. However, soil salinity significantly reduced its growth and yield. Our study investigated whether applying exogenous nitrogen (N) (ammonium nitrate; AN) could improve salinity tolerance in mungbean seedlings by supplementing them with N under different concentrations of NaCl stress. Results revealed that the increased NaCl stress levels significantly reduced shoot height, root length, fresh and dry weight, and chlorophyll pigment. In contrast, increased hydrogen peroxide (H2O2), malondialdehyde, organic solutes (soluble protein, proline, and soluble sugar), antioxidant enzymes, i.e., superoxide dismutase, ascorbate peroxidase, catalase, and peroxidase, and abscisic acid were significantly elevated. Jasmonic acid (JA) increased at 40 mM stress and decreased at 120 mM stress level. There was also a significant reduction in the concentration of cytokinin, indole acetic acid, and N-metabolizing enzymes, viz. nitrate reductase (NR) and glutamate synthase (GOGAT) activity. In contrast, N supplementation reduced H2O2 and MDA but improved chlorophyll levels, osmolytes, phytohormones, N assimilation potential, and enzymatic antioxidant activity. Exogenous N supplementation reduced the impairments by enhancing their growth and metabolism. Conclusively, AN application may provide an effective strategy for improving salinity tolerance and mitigating damage caused by salinity by modulating physiological responses.

Keywords: Abiotic stress; salinization; nitrogen fertilization; antioxidant mechanism; salt-sensitivity;

1. Introduction

The mung bean is an economically significant crop species rich in minerals, protein, vitamins, fibers, essential amino acids, and carbohydrates (Sehrawat et al. 2019). Globally, mungbean accounts for approximately 5% of all pulse production (World Vegetable Centre 2018). Mung bean plants are impacted by salinity in several ways, viz seed germination, growth characteristics, relative water content (RWC), nutrient acquisition, excessive reactive oxygen species (ROS), photosynthesis, nodulation, and nodule respiration. It has also been reported that under 50 mM NaCl, its yield decreased to almost 70%, adversely affecting crop quality (Sehrawat et al. 2019). Hence, it is imperative to improve salt tolerance to maintain its productivity in salinity-affected soils worldwide.

Increasing soil salinity generally causes a higher accumulation of toxic salt ions, including namely Na^+ and Cl^-, leading to ionic toxicity, osmotic imbalance, impaired nutrient acquisition, metabolic disruption, excessive ROS, and cellular oxidative stress damage (Ullah et al. 2022a; Wang et al., 2025). It is evident that plants adopt several mechanisms to protect themselves from the adverse effects associated with salinity, including the production of osmolytes, the exclusion or compartmentalization of Na^+ and Cl^- into vacuoles and old tissues, the biosynthesis and regulation of antioxidant mechanisms (non-enzymatic and enzymatic) (Solangi et al. 2019; Zaid and Wani 2019). As a consequence of excessive salinity causes ionic toxicity and osmotic stress effects, resulting in the generation of ROS such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\bullet-}$), and hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) (Ullah et al. 2022b). The higher ROS accumulation causes oxidative stress, leading to metabolic changes and degradation of cellular membranes (lipid peroxidation), RNA, DNA, and proteins (Mignolet-Spruyt et al. 2016; Azeem et al. 2020; Zhang et al. 2020).

When plants undergo oxidative stress, one of their immediate responses is to eliminate excess ROS via physiological and molecular mechanisms that stimulate antioxidant enzymes

viz. peroxidase (POD), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) and antioxidant metabolites (Sarker and Ercisli 2022; Sarker et al. 2023), that remove ROS. Therefore, plants can reduce oxidative stress damage by upregulating their antioxidant system to neutralize ROS and continue average growth. Hence, the upregulation of the antioxidant mechanism has been reported to correlate positively with the salt stress condition in the soil (Wang et al., 2017; Sahab et al. 2021). In stressful conditions, plants accumulate salt-relieving solutes such as soluble sugars, glycine betaine, and proline. To maintain salinity homeostasis, osmolytes regulate water balance and cell division, prevent ion toxicity and chlorophyll degradation, stabilize cellular structures, and eliminate excess ROS (Ullah et al. 2020; Singh et al. 2022).

Phytohormones (PGRs) are natural signalling molecules that act as plant growth regulators (PGRs). As phytohormone signalling pathways cross-talk extensively, phytohormones can have several effects on metabolic and signalling pathways, such as additive, synergistic, and antagonistic effects (Aerts et al., 2021), and they can also have significant effects on plant responses to biotic and abiotic stresses (Peleg and Blumwald 2011; Wei et al. 2021). A deeper understanding of phytohormone signaling pathways regulating salt stress responses may provide a better understanding of abiotic stress responses and the interplay between phytohormones, potentially revealing new strategies to enhance salt stress adaptation in crop species.

Nitrogen (N) is an essential constituent of organic molecules, and in the process of N metabolism, nitrate reductase (NR) and the reductase (NR) function to reduce absorbed nitrate (NO_3^-) into nitrite (NO_2^-) and then into ammonia (NH_4^+) (Liu et al. 2014). Following its synthesis, NH_4^+ is converted into amino acids either through the action of glutamine synthetase and glutamate synthase (GS/GOGAT) or through another enzyme known as glutamate dehydrogenase (GDH) (Khan et al. 2023). The increase in salinity has adverse effects on N metabolism

due to reduced uptake of NO_3^- and NH_4^+ or decreased activity of N-metabolizing enzymes (Ullah et al. 2019, 2022a; Abile et al., 2024).

Salinization affects ≈3600 Mha of arable land annually, causing USD 27.5 billion in losses (Zhang and Shi 2013; Wani et al. 2020). Humanity's significant challenge is the rapid rise in food demand and soil salinization. Besides, rapid industrialization, global warming, environmental fluctuations, irrigation with salty water, and unsustainable fertilizer consumption will further exacerbate the future salinization problem (Zhu 2001; Zuo et al. 2021; Ma et al. 2022). So far, researchers have suggested several management strategies for reducing the adverse impacts of salinity on plants, including cultivating salt-tolerant varieties and improving soil salinity. Nevertheless, the implementation of these measures will take a very long time. In previous studies, mineral nutrition and supplemental plant growth regulators are the most effective methods to improve plant tolerance (Singh et al. 2014; Guo et al. 2017; Ravi et al. 2022; Raveena et al. 2024).

It is well-documented that N assimilation plays a vital role in determining a plant's ability to withstand salinity. However, a complex relationship exists between N metabolism and salinity due to several factors, such as salt stress intensity, plant species' susceptibility, and soil availability (Dai et al. 2015). Besides being a vital macronutrient, N is a key player contributing to better crop production after water (Kant 2018). Further, N participates in many cellular functions within plants owing to its presence in enzymes, proteins, amino acids, nucleic acids, osmolytes, and hormones (Singh et al. 2014, 2019; Liu et al. 2023). all of which function differently and are involved in salinity tolerance mechanisms (Arghavani et al. 2017).

Thus, this study speculates that additional N applications will increase mungbean salt tolerance. Exogenous N has been shown to significantly modulate the growth of Brassica (Siddiqui et al. 2010), wheat (Arghavani et al. 2017), tomato plants (Singh et al. 2019), and

cotton (Sikder et al. 2020), subjected to salt stress. Based on the findings of the studies discussed above, N-applied positive modulation occurred via the upregulation of photosynthesis and antioxidant systems and the reduction of oxidative stress damage. Despite numerous studies investigating the effects of salinity on mung beans, little is known about the interaction between salinity stress and exogenous N application in mung beans. Accordingly, this study investigated the role of N in improving mungbean salt tolerance. Our study hypothesized that (a) increasing salinity could adversely affect mungbean plants by disturbing their physio-biochemical functions and growth, and (b) exogenous N supplementation could reduce salinity-stress-induced damages in mungbeans by modulating their physio-biochemical characteristics. A comprehensive study of morphological and physio-biochemical changes in mungbean plants was conducted to test our hypothesis, including a detailed analysis of the changes in shoot and root biomass, photosynthetic pigments, salt ion concentration, antioxidant mechanisms, osmolytes accumulation, endogenous phytohormones, and N metabolism under salinity and N supplementation.

2. Materials and Methods

2.1. Study area and experimental steps

A greenhouse experiment was conducted at the campus of Jiangsu University, Zhenjiang City, China. Two kilograms of soil (Silt-loamy soil; pH 6.9 and EC 0.288 dS/m) were placed in small plastic pots with a bottom opening (2 cm in diameter). Following a complete randomized block design (CRBD), pots containing uniform seedlings (n = 2 seedlings per pot) were selected and divided into eight groups for salt stress treatments and N application. Four treatments were conducted for salinity stress (0 mM, 40 mM, 80 mM, and 120 mM NaCl). The four remaining groups were subjected to the same salinity stress levels but supplied with exogenous N. A solution of ammonium nitrates i.e., during irrigation, 5 mM of ammonium/ni-

trate (50/50 ratio) was applied to the root medium. Ammonium sulfate ($(NH_4)_2SO_4$) and calcium nitrate ($Ca(NO_3)_2$) were used as a source of NH_4^+ and NO_3^- (50/50) and were mixed with water (-AN, +AN) and applied to the pots during irrigation. The vapor-transpired water was replenished every three days to maintain the target volume. Following is the formula for calculating soil relative water content (SRWC):

$$SRWC = ([(Wsoil - Wpot - DWsoil) / ((WFC - Wpot - DWsoil)] * 100)$$

In this case, Wsoil corresponds to the weight of the soil, pot, and water at the time of saturation; Wpot corresponds to the weight of the empty plastic pot; DW soil corresponds to the weight of the dry soil; and WFC corresponds to the weight of the soil at capacity (soil + pot + water). Each treatment was replicated five times. The growth characteristics of the mungbean plants were determined six weeks after harvesting. The remaining pots were harvested and frozen in liquid N the same day before being stored at -80 °C for laboratory analysis.

2.2. Determination of growth characteristics

Root length, shoot height, fresh weight (FW), and dry weight (DW) were measured after the plants were harvested. Carefully uprooted the plants and divided them into different parts. The FW and length of the root and shoot were calculated. After this, keep the samples at 60 °C constant temperature in the oven to dry for about three to four days. Lastly, dry weight was calculated.

2.3. Determination of chlorophyll pigments

A chlorophyll extract (0.1 to 0.3 g) was prepared using ethanol (95 %, v/v), and the absorbances were measured at 665 nm and 649 nm (Lichtenthaler and Buschmann 2001).

2.4. Determination of mineral elements

Leaf powder samples were digested with concentrated HNO_3 and a volume of 15 ml of deionized water. An inductively coupled plasma-optical emission spectrometry (ICP-OES) was used to estimate Na^+ and K^+ contents (Yousfi et al. 2010).

2.5. Determination of phytohormones

We determined the endogenous phytohormones, including abscisic acid (ABA), indole acetic acid (IAA), cytokinin (CKT), and jasmonic acid (JA) in fresh leaf samples using ELISA according to the instructions included with the Plant ABA ELISA Kit, Plant IAA ELISA Kit, Plant JA ELISA Kit, and Plant CTK ELISA Kit (MiBio, Shanghai).

2.6. Oxidative stress indicators

A standardized technique was used to measure the concentration of H_2O_2 (Patterson et al. 1984). Fresh samples (0.2 g) were homogenized in trichloroacetic acid (5 ml, 0.1%) and then centrifuged at 5000 × *g* (10 min, 4 ˚C). An absorbance measurement was conducted at 410 nm (Patterson et al. 1984). MDA content was measured using the thiobarbituric acid (TBA) test (Zhou et al. 2007). Leaf samples (0.5 g) were homogenized in trichloroacetic acid (TCA; 1 ml and 5%) solution and then centrifuged at 5,000 × *g* (4◦C, 10 min). The OD450, OD532, and OD600 nm were measured using a spectrophotometer

2.7. Determination of antioxidant enzyme activities

We ground and homogenized fresh leaf samples in phosphate buffer (0.1 M; pH 7.3) containing EDTA (0.6 mM). We centrifuged the mixture at 12000 × g (10 min; 4°C). Next, the enzyme extract in the supernatant was used for the assays. The catalase (CAT) activity was assessed by monitoring the disappearance of H_2O_2 (Sabra et al. 2012), and the absorbance was read at 240 nm using a spectrophotometer. The absorbance was read at 460 nm for peroxidase (POD) estimation following standard methods (Wang et al. 2018). Moreover, using a spectrophotometer, we measured the superoxide dismutase (SOD) activity based on the decline rate of nitroblue tetrazolium at 560 nm (Giannospolitis and Ries 1977). The ascorbate peroxidase (APX) activity was measured following a standard method (Iqbal et al. 2024a). A rise in absorption (290 nm) was noted following ascorbate oxidation using a spectrophotometer.

2.8. Determination of proline and soluble sugar, soluble protein, and glycine betaine

After homogenizing 0.3 g of samples in sulfosalicylic acid (3%; 5 ml) and acetic acid (10%; 2 ml), they were centrifuged at 5,000 × *g* (10 min). The absorbance at 520 nm was measured using a spectrometer (Liu et al. 2014). We calculated the soluble sugar using the anthrone sulphuric acid (Irigoyen et al. 1992). Leaf samples were extracted in distilled water and centrifuged to determine the concentration of glycine betaine. The absorbance at 530 nm was measured using a spectrophotometer (Grieve and Grattan 1983). Using glucose as a standard, we measured the absorbance spectrophotometrically at 620 nm. Following Bradford's (Bradford 1976), we determined the soluble protein concentration using the Coomassie brilliant blue G-250 reagent.

2.9. Determination of N metabolizing enzymes

The resultant supernatant assessed the NR activity using a diazo-coupling technique with Griess reagent (Sánchez-Rodríguez et al. 2011). In addition, GS activity was determined according to a previous study (Ullah et al. 2019). The NADH oxidation was continuously monitored by measurement of absorbance at 340 nm using a spectrophotometer.

2.10. Statistical analysis

The measurements were repeated three times. Statistical analysis and one-way analysis of variance (ANOVA) were performed using SPSS version 16.0 (Chicago, IL, United States). We used Duncan's multiple range tests at a significance level of $p > 0.05$ to compare the mean values. The figures were created using Microsoft Excel 2013. Pearson correlation and principle component analysis were carried out using OriginPro 2023.

3. Results

3.1. Hazardous effects of salinity on growth attributes of Vigna radiata L. and its alleviation by N-application

The results showed that salinity stress substantially reduced the growth parameters of the *Vigna radiata* L crop. The plant shoot height, shoot FW (fresh weight), shoot DW (dry weight), root length, root FW, and root DW experienced a 31, 38, 32, 26, 67, and 42% decline under 40 mM, while 37, 55, 62, 35, 74, and 71% at 80 mM, and 54, 62, 71, 48, 77, and 71% inhibition under 120 mM salinity stress compared to control treatment, respectively (Fig. 1A-F). However, the N-application improved the growth indices such as shoot height, shoot FW, shoot 2.4, 2.1, 1.4, 2.9, and 2.4% at 80 mM, while 2.4, 3.0, 1.8, 1.8, 5, and 4% at 120 mM of salinity stress, respectively (Fig. 1A-F).

3.2. Interactive effect of salinity stress and N-application on Vigna radiata L chlorophyll pigments.

The physiological attributes are significantly reduced under salinity stress. Therefore, Chl a and Chl b declined up to 13.2 and 35% at 40 mM, 58 and 55% at 80 mM, and 41 and 63% at 120 mM of salinity stress, respectively, compared to the control treatment. Interestingly, N-application did not significantly affect Chl a concentration under either control or salinity stress treatments (Fig. 2A-B). Although N-application improved Chl b concentration under 40 mM stress, it had no significant impact under 80 mM and 120 mM stress levels. Additionally, the ratio of Chl a/Chl b increased across all salinity treatments compared to the control. However, N-application had no significant effect on the Chl a/Chl b ratio under salinity treatments compared to their untreated counterparts.

3.3. Increment in nutrient uptake b N-application under salt stress

The K^+ and K^+/Na^+ reduced up to 27 and 52% at 40 mM; 41 and 71% at 80 mM, whereas 49- and 80% declined following 120 mM of salt stress, respectively (Fig. 3AC). Contrary to others, Na^+ uptake significantly increased by 1.5-, 2.0-, and 2.5-fold under all salinity levels compared to control (Fig. 3B). However, N supplementation decreased Na^+ by 1.4-fold at 40 mM, 1.7-fold at 80 mM, and 2.7-fold at 120 mM salinity, respectively (3B). Furthermore, the results revealed that N application enhanced the concentration of K^+ and K^+/Na^+ by 1.1-, 1.6-fold at 40 mM, 1.1-, and 2.6-fold at 80 mM, while 1.6-, and 4.4-fold at 120 mM of salinity, respectively (3AC).

3.4. Changes in endogenous phytohormones

The concentration of abscisic acid (ABA) increased by 6.0, 21.7, and 47.9% at 40, 80, and 120 mM salinity stress, whereas jasmonic acid (JA) enhanced at 40 mM by 3.3%, compared to controlled conditions (Fig. 4 A-B). In contrast, indole acetic acid (IAA) and cytokinin (CKT) decreased by 31.8 and 23.8 at 40 mM, 30.2 and 36.5% at 80 mM, and 42.2, and 33.8% at 120 mM salinity stress, respectively (Fig 4 C-D). Similarly, compared to their controlled conditions, JA decreased by 7.2 and 36.0% at 80 and 120 mM Salinity stress levels. Exogenous N supplementation improved the phytohormone concentrations. For instance, the concentrations of JA, IAA, ABA, and CTK enhanced by 5, 13.4, 6.3, and 12.5% at 40 mM, and 4.3, 21, 1.1, and 17% at 80 mM, and 7, 11, 6, and 14% at 120 mM, compared to the control treatments (Fig. 4 A-D). In conclusion, jasmonic acid (JA) increased at 40 mM stress and decreased at 120 mM stress level.

3.5. Mitigation of Oxidative Stress Through N-Application Under Saline Conditions

Under salinity stresses, *Vigna radiata* L. plants produced a considerable amount of reactive oxygen species. The H_2O_2 and MDA contents increased by 1.3 and 1.4-fold at 40 mM, 1.9, and 1.5-fold under 80 mM, and 2.4 and 2.3-fold following 120 mM of salinity stress compared

to the control treatment (Fig. 5 A-B). Additionally, the N-application decreased the concentration of H_2O_2 and MDA under controlled and salinity stress treatments compared to their unfertilized pots. Additionally, the antioxidant enzymes such as SOD, CAT, POD, and APX improved by 1.1, 1.2, 1.05, and 1.2-fold at 40 mM, 1.3, 1.4, 1.4, and 1.3-fold at 80 mM, whereas 1.4, 1.46, 1.5, and 1.4-fold at 120 mM of salinity stress, respectively (Fig. 5 C-F). Exogenous N supplementation further strengthened the enzymatic antioxidant mechanism. For instance, the N-application caused an increment of 1.1, 1.36, 1.2, and 1.15-fold at 40 mM, 1.3, 1.3, 1.5, and 1.2-fold at 80 mM, while 1.4, 1.5, 1.6, and 1.3-fold at 120 mM of salinity stress was noticed, respectively (Fig. 5 C-F).

3.6. The negative impact of salinity on biochemical changes and its mitigation by N-application

The metabolites study showed a different pattern since proline, protein, and sugar contents were enhanced by 2.21-, 1.35-, and 1.08-fold at 40 mM of salt stress. Meanwhile, proline and protein improved 3.2- and 1.6-fold at 80 mM and 1.6- and 1.1-fold under 120 mM salinity stress compared to the control treatment (Fig. 6 A-C). Furthermore, the sugar content faced a significant reduction at 80 and 120 mM in salt stress compared to the control. Like salt stress, the N-application improved the proline and protein content by 2.3- and 1.2-fold at 40 mM, whereas 2.6- and 1.1-fold at 80 mM salinity stress, respectively (Fig. 6 A-B). On the other hand, at 120 mM of salt stress, N-application had no significant effect on proline and protein concentration (Fig. 6 B). The application of N improved soluble sugar by 1.21 and 1.1-fold under control and 80 mM stress, respectively (Fig. 6 C). The concentration of GB decreased by 21.3, and 36.5% at 80 and 120 mM salinity stress levels compared to the control treatments (Fig. 6 D). In contrast, N supplementation improved the GB concentration by 1.27, 1.54, and 1.11-fold at 40, 80, and 120 mM stress levels compared to their untreated peers (Fig. 6 D).

298 *3.7. Interactive impact of salinity and N-application on N metabolism*

299 The $NO_3^-$and NH_4^+ reduced up to 17 and 17% at 40 mM, while 60 and 44% at 80 mM, whereas
300 67 and 72% declined following 120 mM of salt stress, respectively (Fig. 7 A-B). Furthermore,
301 the results revealed that N-application enhanced the nutrient uptake, including $NO_3^-$and NH_4^+,
302 by 1.2-, 1.2-fold at 40 mM, 2.6-, and 1.8-fold at 80 mM salinity. Moreover, the NR and GS
303 significantly reduced under all stress levels (Fig 7 C-D). Compared to the control treatment,
304 both parameters were reduced to 21-and- 22% at 40 mM, 32- and 43% at 80 mM, and 62 and
305 63% at 120 mM of salinity stress. Similarly, N-application significantly enhanced the NR
306 under control treatments (1.4-fold) compared to its untreated peer. Meanwhile, GS activity
307 increased by 1.10, 1.27, and 2.1-fold at control, 40 mM, and 1.20 mM treatments, respectively
308 (Fig. 7 C-D).

309 *3.8. Correlation analysis and principal component analysis*

310 The correlation analysis revealed that SHL (shoot height length), SHFW (shoot fresh
311 weight), SHDW (shoot dry weight), RTL (root length), RTFW (root fresh weight), and RTDW
312 (root dry weight) exhibited a significant positive correlation ($p < 0.05$) with photosynthetic
313 pigments such as Chl a, Chl b, and the Chl a/Chl b ratio, as well as with osmoregulatory
314 compounds like proline and glycine betaine (Fig. 8). These growth parameters also positively
315 correlated with enzymes associated with N metabolism, including NR and GS, and N forms
316 such as NO_3^- and NH_4^+. On the contrary, a significant negative correlation was observed
317 among antioxidant enzymes, including SOD, POD, CAT, and APX, with N assimilation en-
318 zymes (NR and GS). Moreover, H_2O_2 and MDA, oxidative stress markers, displayed a signif-
319 icant negative correlation with photosynthetic pigments and growth parameters, indicating
320 that higher stress markers are associated with lower growth and pigment levels. Additionally,
321 the plant hormones ABA showed a significant negative correlation with the growth parameters

and photosynthetic pigments (Fig. 8). At the same time, JA, IAA, and CTK exhibited a strong positive correlation, suggesting their role in promoting growth and pigment synthesis (Fig. 8).

4. Discussion

The seedling stage in a crop's life cycle is vital and particularly vulnerable, and vigorous seedlings assure a high yield. At the early stage of rice seedlings, salt stress causes significant reductions in root number, root length, and shoot length (Hussain et al. 2017). Likewise, another experiment demonstrated that tomato seedlings treated with salt experienced a significant decline in growth attributes (such as plant height, reduced biomass, and reduced amounts of photosynthetic pigments) and gross photosynthesis (Singh et al. 2019). Similarly, we observed that the root-shoot length, fresh and dry weight as well and chlorophyll pigments significantly decreased when salinity stress was increased, which could be directly correlated with a proportionally increased cellular Na^+ (Ullah et al. 2022a), H_2O_2 and MDA (Fig. 4A-B). Supplemental N improved growth and reduced NaCl-associated toxicity by increasing chlorophyll pigments, K^+, and the K^+/Na^+ ratio and enhancing N-metabolism and antioxidant activity.

Moreover, mungbean growth and biomass were improved by N supplementation under controlled conditions compared with untreated plants, indicating the high N demands of young seedlings with a more significant improvement in physiological and biochemical production. The biological systems of plants require adequate amounts of N for a wide range of vital metabolic functions (Gong et al. 2013; Raveena and Surendran, 2023). Consequently, N availability is central in regulating physiological mechanisms under unstressed and stressed conditions, including salinity. Excessive salinity leads to an imbalance of cellular ions, which leads to excess production of ROS, including H_2O_2. Under stressful environments, such as salinity, MDA is commonly used to assess cellular oxidative damage (Saidimoradi et al. 2019). As salinity stress levels increased, we noticed increased H2O2 and MDA contents in mung bean

leaves. Our findings suggest that ROS interacts with macromolecules (proteins and lipids) and critical metabolic enzymes, leading to the degradation of photosynthetic performance and decreased mungbean plant growth and biomass. Under salinity stress, N supplementation to mungbean plants significantly reduced H_2O_2 and MDA contents, suggesting N can help protect membranes (Singh et al. 2019; Sikder et al. 2020).

Scavenging ROS during stressful conditions is essential for preventing oxidative damage (Zhao et al. 2022). In plants, antioxidant mechanisms enable them to eliminate ROS and minimize or prevent damage caused by ROS to cells (Li et al. 2024). Considering that N supplementation increases the antioxidant potential and salinity tolerance (Ahanger et al. 2019), we assessed the H_2O_2 and the SOD, APX, POD, and CAT activities. H_2O_2 levels were increased by salinity in comparison with the control. The N supplementation, however, inhibited H_2O_2 levels significantly compared to unfertilized plants. The decline in H_2O_2 and MDA could be attributed to the immediate strengthening of enzymatic antioxidants and osmolytes (such as proline, soluble protein, and soluble sugar) under exogenous N supplementation. It has been shown that N protects against oxidative stress induced by external or internal stresses (Sikder et al. 2020; Singh et al. 2019). The N may have a positive effect due to its role as a component of the antioxidant mechanism. Furthermore, several other studies have found that co-applying N significantly improved antioxidant enzyme levels in tomatoes (Singh et al. 2014), soybean (Borella et al. 2019), blueberry (Yañez-Mansilla et al. 2014), and cotton (Sikder et al. 2020), which corroborates our findings. Moreover, plants must regulate osmotic potential to minimize the adverse effects of osmotic stresses and dehydration. The osmolytes (such as glycine betaine, proline, soluble protein, and soluble sugar) play a critical role in maintaining salt balance, cell division, preventing ion toxicity, chlorophyll degradation, stabilizing cell structures, and eliminating excess ROS (Singh et al. 2022).

We noticed a significant increase in proline and soluble proteins under salinity stress, similar to previous research (Meriem et al. 2014; Sharif et al. 2018; Ullah et al. 2022a). Additionally, N application enhanced osmotic regulation by significantly increasing the osmoregulation substance. For instance, exogenous N supplementation improved the concentration of soluble protein, sugar, and proline. Another researcher observed improvements in relative water content, antioxidant potential, and osmolytes in wheat subjected to salinity, including proline and lower membrane permeability (Agami et al. 2018).

Mungbean plants appear to use osmolytes to combat salinity stress, especially at low and medium stress (40 and 80 mM). The intersectional point is that despite the increased concentration of proline and soluble protein at all stress levels compared to the control, their concentration was more significant at 80 mM (80 > 40 > 120 > 0 mM NaCl). In contrast, the activity of the antioxidant enzyme increased as salinity stress levels (i.e., ascending). Hence, we suggest that mungbean seedlings spend more energy adjusting to osmotic conditions under low-to-medium salinity stress conditions. However, under high salinity stress, they concentrate on antioxidant defence mechanisms. Proline concentrations have been suggested to protect against salt damage/osmotic stress in plants by maintaining cellular osmotic potential. Additionally, they stabilize other functional units, such as membranes, electron transport complex II, enzymes, and proteins (Iqbal et al. 2024a). Salt stress tolerance is boosted by the accumulation of soluble proteins, which can act as osmosis (Zhang et al. 2013; Sarker et al. 2018), corroborates our results. Proline, in addition to its function in osmotic regulation, also plays several other functions, including the scavenging of ROS, stabilizing subcellular structures, and buffering cellular redox potential under salinity stress conditions (Singh et al. 2015; Khanna-Chopra et al. 2019).

The growth-promoting hormone IAA is an important signalling molecule in plants. Auxin has been linked previously to salt stress responses in plants; however, it is unclear how these

responses are controlled (Ryu and Cho 2015). The reduced IAA levels in salinity-treated mungbean plants in our study are consistent with previous findings on different plants species, including tomato (Albacete et al. 2008), Iris hexagona (Wang et al. 2001) and wheat (Shakirova et al. 2003). There may be a connection between this and the simultaneous suppression of growth in leaves and roots found in these studies. CKs also play a role in regulating plant growth and development, similar to auxins. Plant cytokinin levels indicate their ability to resist environmental stresses, making plants more tolerant of abiotic stresses such as high salinity and temperatures (Javed et al. 2023). A study found that increasing the level of CK in cereals improved their resistance to salinity stress (Iqbal et al. 2024b). It has been demonstrated in numerous studies that salt stress can lead to a reduction in overall CK levels (Vanková et al. 2014), which corroborates our findings. ABA promotes the closure of stomata and is a crucial stress indicator and mediator of the stress response in plants. In our study, all stress levels increased ABA content compared to controlled conditions. The increase in ABA levels following adverse environmental conditions has been reported in several previous publications (Albacete et al. 2008; Dobra et al. 2010; Vanková et al. 2014). Few studies have examined the effects of salt stress on endogenous JA levels. Compared to the control condition, the jasmonic content increased remained unchanged and decreased at 40-, 80-, and 120 mM stress levels, respectively. The current study revealed that N fertilization enhanced the concentrations of JA, ABA, CTK and IAA under salinity stress levels compared to their untreated peers. In previous studies, N applications have been found to improve plant growth by regulating endogenous auxin levels (Ye et al. 2021). Plant hormones can be altered by both N and nitrate, which serve as N sources and signal molecules (Garnica et al. 2010; Kumar et al. 2018). Moreover, exogenous N fertilizer enhances the concentration of ABA and IAA in the crops by revealing a more significant fluctuation in growth and productivity (Borella et al. 2019). In conclusion, we suggest that N supplementation could enhance mungbean resistance to salinity

stress by upregulating endogenous phytohormones, leading to improved growth compared to untreated plants.

The regulation of N metabolism plays a vital role in salt stress tolerance through several pathways (Arghavani et al. 2017; Yan et al. 2021). Plants use inorganic N from NO_3^- and NH_4^+ for metabolic processes through their roots (Luo et al. 2013). Increasing salinity stress levels significantly reduced NO_3^- ions in mungbean leaves. This could be attributed to NO_3^- uptake and loading of the root xylem is sensitive to salinity (Debouba et al. 2007). Plants absorb inorganic N, which can be converted into organic N through the action of NR, which is the starting factor and rate-limiting enzyme in the N metabolism. Thus, N accumulation and protein content positively relate to its activity. Through the GS/GOGAT cycle, approximately 95% of NH_4^+ is converted to amino acids. The main form of N in plants and transportation is amino acids. GS is an essential enzyme in this cycle (Giunta et al. 2020).

Moreover, the foliar NR activity declined under increased salinity stress levels. Therefore, the decline in NO_3^- concentration may occur because of reduced NR activity or a direct reduction in enzyme synthesis under increased salinity stress (Iqbal 2018; Ullah et al. 2019). Hence, we suggest that the reduced NO_3^- uptake and accumulation may have affected N metabolism, which could have resulted in a significantly decreased amino acid and protein synthesis, thus considerably reducing the dry weight of mungbean plants under salinity stress (Xie et al. 2020). Further, a decreased NH_4^+ was observed under increased salinity stress. In salt-induced NO_3^- reduction reactions, NH_4^+ cannot be supplied to GS/GOGAT, inhibiting amino acid synthesis (Wang et al. 2012).

Moreover, the GS enzyme decreased with increased salinity stress. Several studies reported salt-induced reductions in plant NH_4^+ through the impaired GS/GOGAT cycle (Wang et al. 2012; Meng et al. 2016; Ullah et al. 2019). Furthermore, a decrease in NH_4^+ accumulation under salinity stress may result from gene downregulation related to the assimilation of NH_4^+

due to NO_3^- deficiency, which reduces N assimilation (Wang et al. 2012). Furthermore, increased salinity stress reduced the ability of NO_3^- to be reduced (or NR- inhibition) and to be assimilated (GS), thus impairing N assimilation and amino acid metabolism and protein synthesis (Ullah et al. 2019). Therefore, biomass growth and accumulation are significantly reduced due to this process. In contrast, N supplementation increased NO_3^-, NH_4^+ concentrations and NR and GS enzyme activities. A recent study demonstrated that N supplementation increases NR activity (Rehman et al. 2020), corroborating our findings. NO_3^- and amino acids are controlled by plant cells through NR, which plays a critical role in the conversion of NO3- to amino acids. Because the availability of NO3 regulates NR activity$^-$, we suggest that an increase in NO_3^- levels might enhance NR activity under N application. Increasing N-metabolizing enzymes can enhance plant N metabolism and facilitate plant protein synthesis and transformation (Chen et al. 2022). The uptake of water and minerals necessary for carbon and N metabolisms enhanced the osmotic regulation of mungbean plants under salinity stress due to the exogenous N supplementation. Therefore, the plants were able to grow under salinity conditions in a relatively normal manner compared to their untreated counterparts.

5. Conclusions

This study demonstrates that nitrogen supplementation effectively mitigates salinity stress in mungbean (*Vigna radiata* L.) through multi-faceted physiological improvements. Under 120 mM NaCl stress, we observed severe growth reductions (shoot-root biomass, 32-42% under 40 mM and 54-71% under 120 mM) that were alleviated by 1.4- 1.8-fold, 2.4- and 4-fold with N application. The treatment enhanced chlorophyll b and improved the K^+/Na^+ ratio (1.6-, 2.6- and 4.4-fold at 40-, 80- and 120 mM, respectively). Moreover, growth hormones such as IAA and CTK improved by 13.4- and 12.5% at 40 mM, 21- and 17% at 80 mM, and 11- and 14% at 120 mM stress, respectively. Notably, N supplementation reduced oxidative stress markers (H2O2/MDA) while boosting antioxidant enzymes (SOD/CAT/POD increased 1-,

1.36-, and 1.2-, at 40 mM, 1.3-, 1.3-, and 1.5-, at 80 mM, while 1.4, 1.5, and 1.6-fold at 120 mM, respectively) and N metabolism. These findings establish that N application prioritizes metabolic repair over stress defense, offering a practical solution (50 mg/kg soil N) for saline agriculture. These findings establish a physiological framework for developing climate-resilient mungbean cultivation strategies in salinity-prone regions while highlighting the need for crop-specific nutrient management approaches in legume production systems. Future studies should elucidate the molecular mechanisms underlying nitrogen-mediated salinity stress tolerance in mungbean, particularly focusing on nutrient-hormone crosstalk and stress adaptation. Moreover, field validation of optimized nitrogen supplementation regimes (timing, dosage, and formulation) under variable climatic conditions is essential for practical implementation.

Author contributions

Fu Shilin: Writing original draft; Software; Data curation; **Babar Iqbal**: Conceptualization; Funding acquisition; Investigation; **Tahani Awad Alahmadi**: Data curation; Formal analysis; Software; **Mohammad Javed Ansari**: Data curation; Writing - review & editing; **Arsahd Jalal**: Formal analysis; Writing - review & editing; **Muhammad Junaid Nazir**: Formal analysis; Software; **Guanlin Li**: Writing - review & editing; Funding acquisition; **Daolin Du**: Resources; Writing - review & editing. All authors contributed to the article and approved the submitted version.

Declarations

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Data availability

All data are included in this article.

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Figures

Figure 1. Changes in growth feature (A) shoot height, (B) shoot fresh weight, (C) shoot dry weight, (D) root length, (E) root fresh weight, and (F) root dry weight, following salinity stress and N supplementation. The bars represent the mean data (n = 3) and SD. T1, T2, T3 and T4 represent 0, 40, 80, and 120 mM salinity stress, respectively. Following the LSD Tukey test, different letters indicate significant differences at $P < 0.05$.

Figure 2. Changes in growth feature (A) Chl a, (B) Chl b, and (C) Chl/Chlb, following salinity stress and N supplementation. The bars represent the mean data (n = 3) and SD. T1, T2, T3 and T4 represent 0, 40, 80, and 120 mM salinity stress, respectively. Following the LSD Tukey test, different letters indicate significant differences at $P < 0.05$.

Figure 3. Changes in the potassium (K^+) concentration (mg g^{-1}) (A), sodium (Na^+) concentration (mg g^{-1}) (B), and K^+/Na^+ ratio (C) under salinity stress and AN application. The bars represent the mean data (n = 3) and SD. T1, T2, T3 and T4 represent 0, 40, 80, and 120 mM salinity stress, respectively. Following the LSD Tukey test, different letters indicate significant differences at $P < 0.05$.

Figure 4. Changes in the concentration of (A) ABA, (B) JA, (C) IAA and (D) CTK following salinity stress and N supplementation. The bars represent the mean data (n = 3) and SD. T1, T2, T3 and T4 represent 0, 40, 80, and 120 mM salinity stress, respectively. Following the LSD Tukey test, different letters indicate significant differences at $P < 0.05$.

Figure 5. Changes in the concentration of (A) H_2O_2, and (B) MDA, and enzymatic acitiity of (C) SOD, (D) CAT, (E) POD, and (F) APX following salinity stress and N supplementation. The bars represent the mean data (n = 3) and SD. T1, T2, T3 and T4 represent 0, 40, 80, and 120 mM salinity stress, respectively. Following the LSD Tukey test, different letters indicate significant differences at $P < 0.05$.

Figure 6. Changes in the concentration of (A) Proline, (B) Soluble protein, (C) Soluble sugar, (D) Glycine betaine following salinity stress and N supplementation. The bars represent the mean data (n = 3) and SD. T1, T2, T3 and T4 represent 0, 40, 80, and 120 mM salinity stress, respectively. Following the LSD Tukey test, different letters indicate significant differences at $P < 0.05$.

Figure 7. Changes in the concentration of (A) NO_3^-, (B) NH_4^+, and enzymatic activities of (C) NR, and (D) GS following salinity stress and N supplementation. The bars represent the mean data (n = 3) and SD. T1, T2, T3 and T4 represent 0, 40, 80, and 120 mM salinity stress, respectively. Following the LSD Tukey test, different letters indicate significant differences at $P < 0.05$.

Figure 8. Analysis of correlations between all parameters examined. The colors blue and red indicate negative and positive correlations, respectively. Shoot height (SHL), shoot fresh weight (SHFW), shoot dry weight (SHDW), root length (RTL), root fresh weight (RTFW), root dry weight (RTDW), sodium (Na^+), potassium (K^+), chlorophyll a (Chl a), chlorophyll b (Chl b), chlorophyll a/chlorophyll b ratio (Chl a/Chl b), soluble sugar (SS), sodium/potassium ratio (Na^+/K^+), proline (Pro), soluble protein (SP), nitrate reductase (NR), glutamine synthetase (GS), nitrate (NO_3^-), ammonium (NH_4^+), ascorbate peroxidase (APX), and superoxide dismutase (SOD), malondialdehyde (MDA), hydrogen peroxide (H_2O_2), peroxidase (POD), catalase (CAT), including abscisic acid (ABA), indole acetic acid (IAA), cytokinin (CKT), and jasmonic acid (JA).

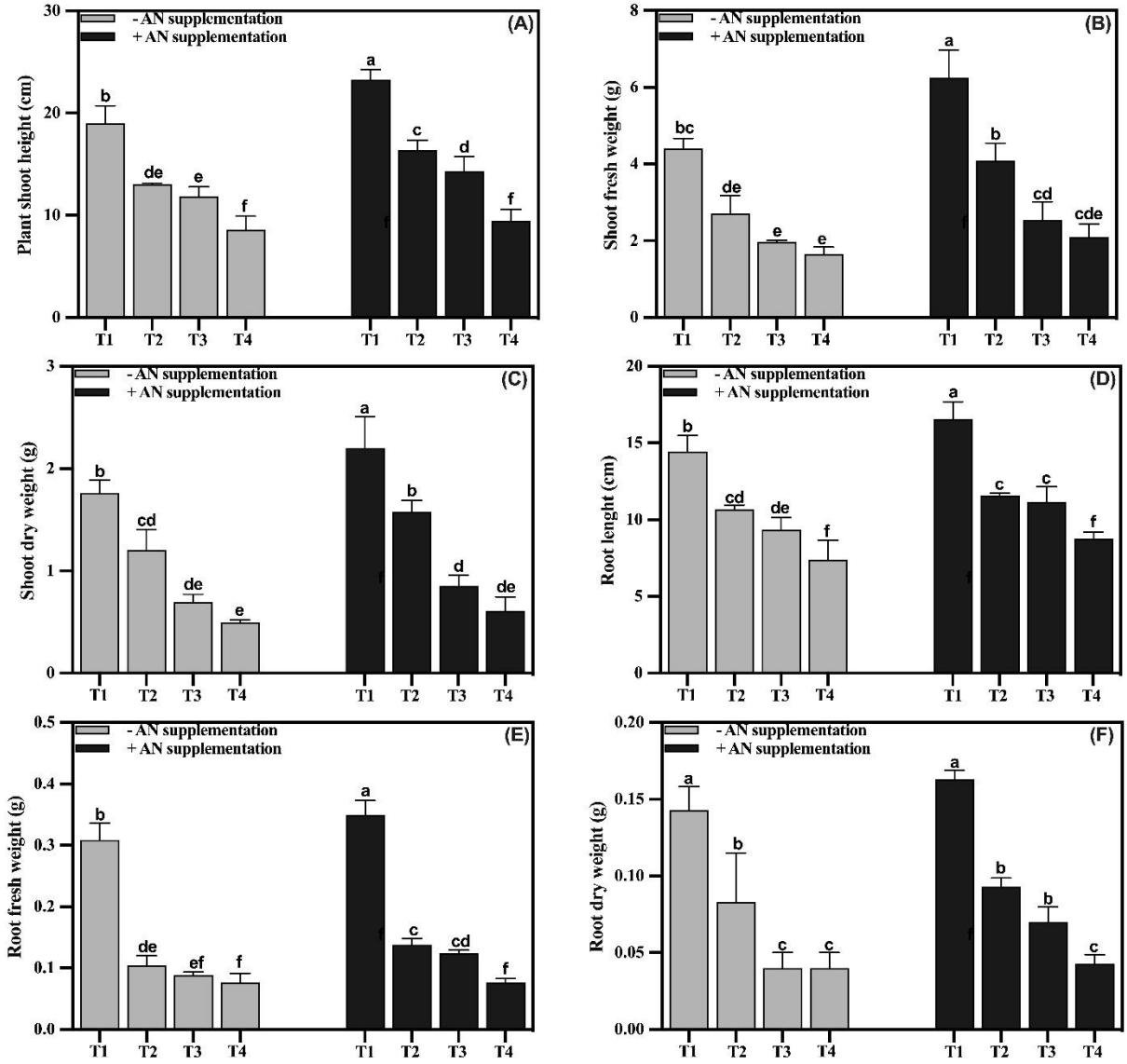

Fig. 1

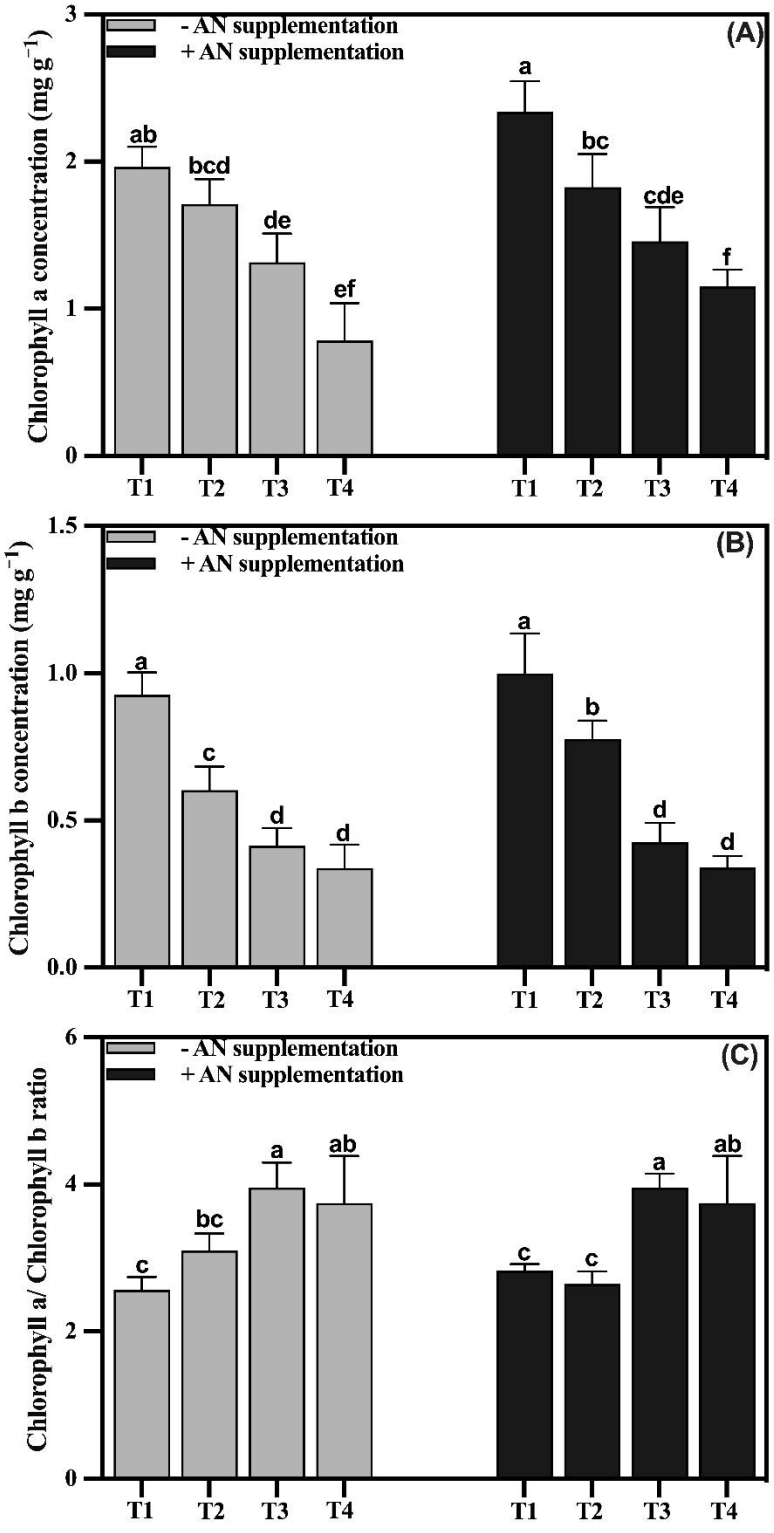
- AN supplementation
+ AN supplementation
(A)
Chlorophyll a concentration (mg g^{-1})
3
2
1
0
ab
bcd
de
ef
a
bc
cde
f
T1
T2
T3
T4
T1
T2
T3
T4
- AN supplementation
+ AN supplementation
(B)
Chlorophyll b concentration (mg g^{-1})
1.5
1.0
0.5
0.0
a
c
d
d
a
b
d
d
T1
T2
T3
T4
T1
T2
T3
T4
- AN supplementation
+ AN supplementation
(C)
Chlorophyll a/ Chlorophyll b ratio
6
4
2
0
c
bc
a
ab
c
c
a
ab
T1
T2
T3
T4
T1
T2
T3
T4

Fig. 2

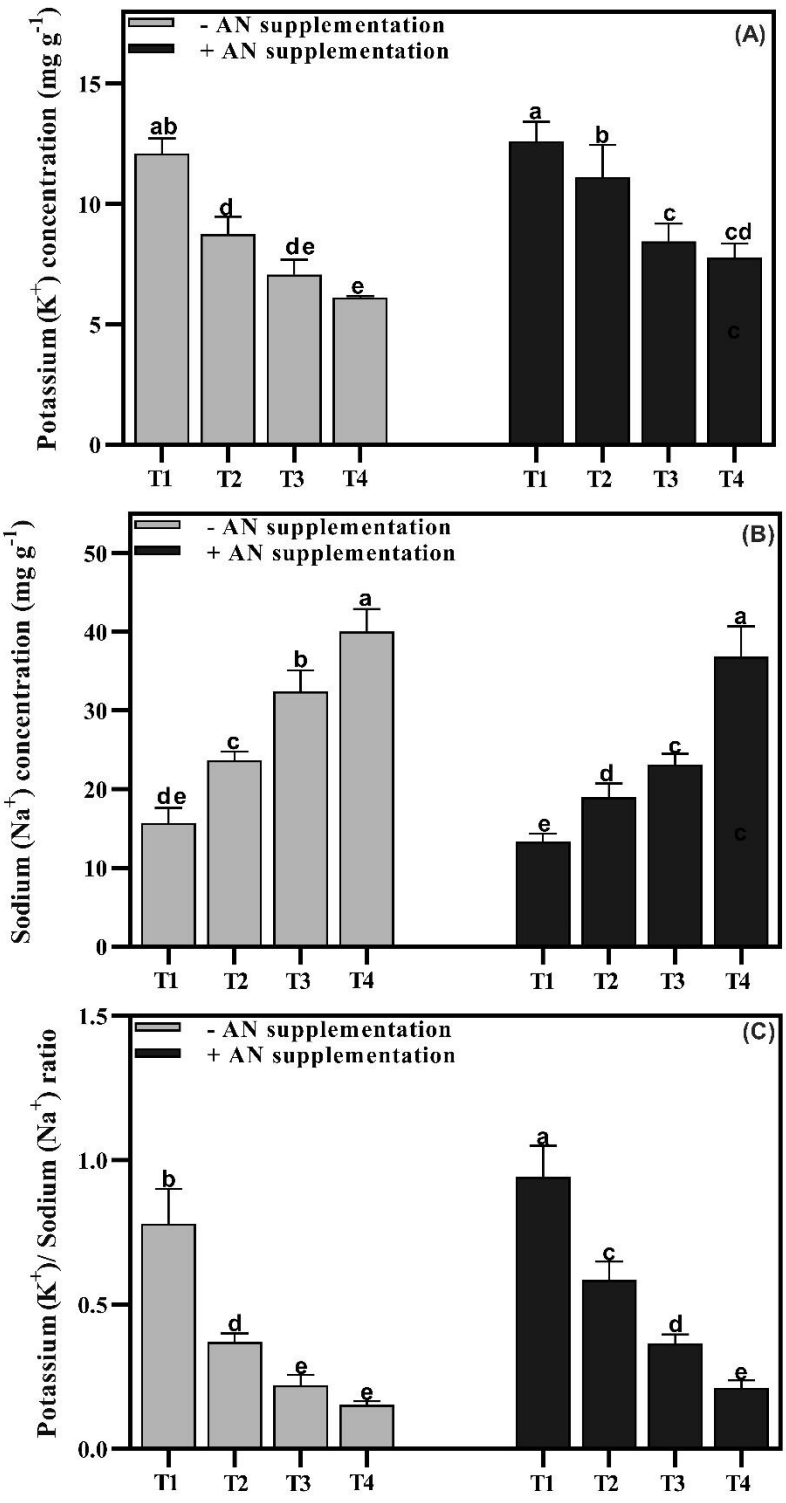
- AN supplementation
+ AN supplementation
(A)
Potassium (K^+) concentration (mg g^{-1})
15
10
5
0
ab
d
de
e
a
b
c
cd
c
T1
T2
T3
T4
T1
T2
T3
T4
- AN supplementation
+ AN supplementation
(B)
Sodium (Na^+) concentration (mg g^{-1})
50
40
30
20
10
0
de
c
b
a
e
d
c
a
c
T1
T2
T3
T4
T1
T2
T3
T4
- AN supplementation
+ AN supplementation
(C)
Potassium (K^+)/ Sodium (Na^+) ratio
1.5
1.0
0.5
0.0
b
d
e
e
a
c
d
e
T1
T2
T3
T4
T1
T2
T3
T4

Fig. 3

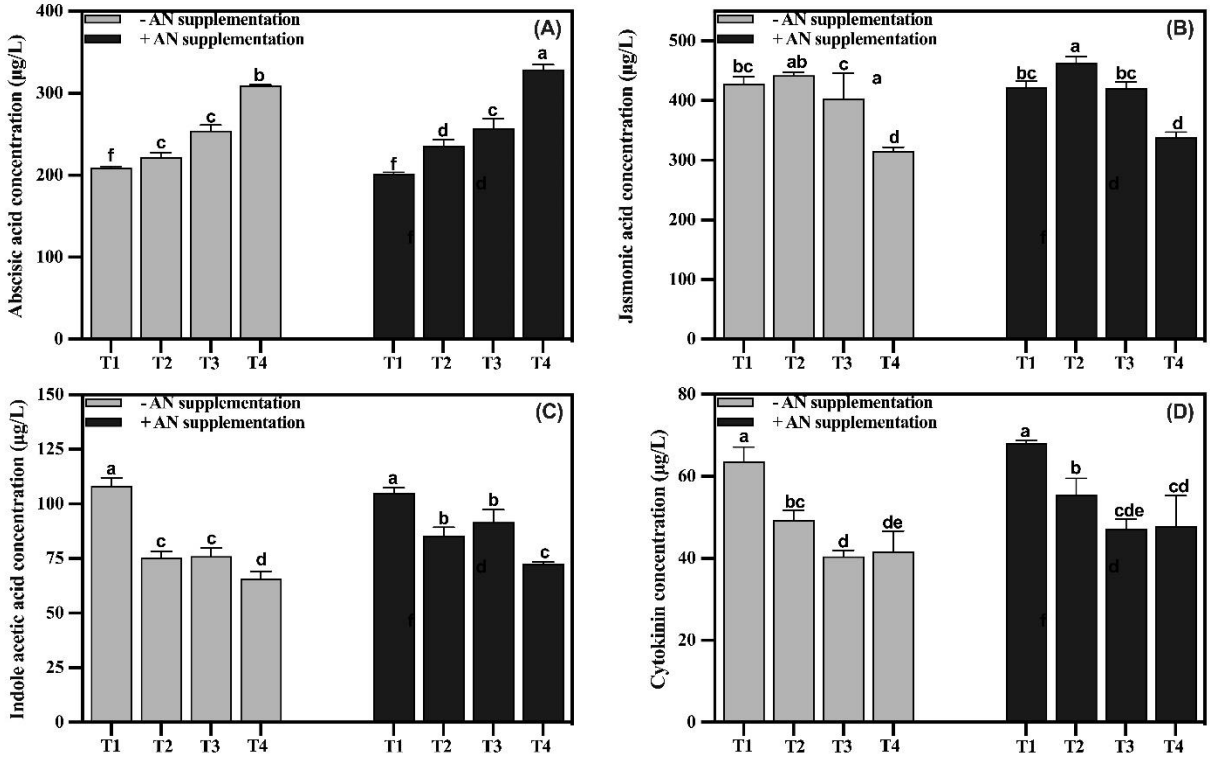

Fig. 4

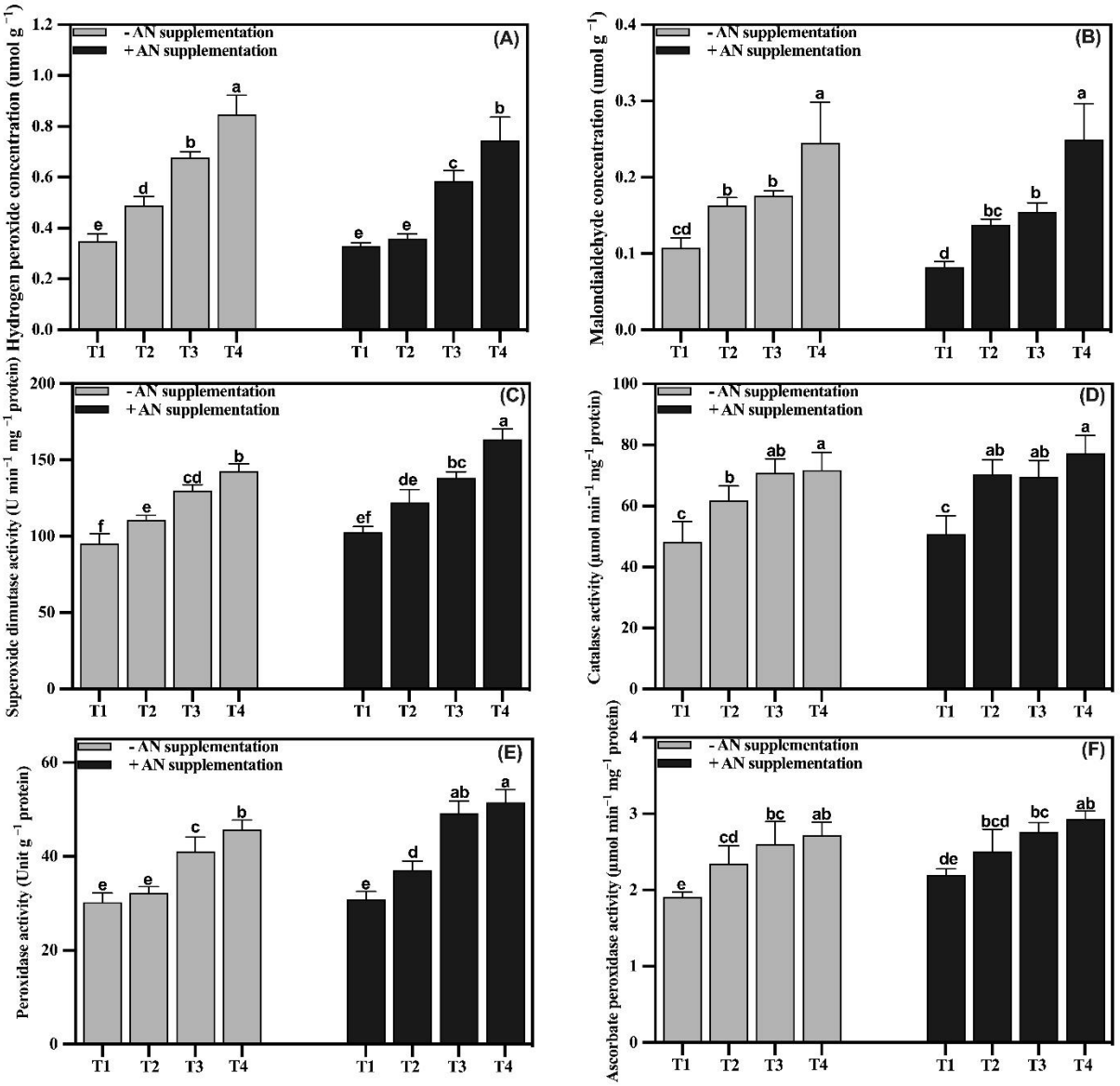
- AN supplementation
+ AN supplementation
(A)
Hydrogen peroxide concentration (nmol g⁻¹)
(B)
Malondialdehyde concentration (umol g⁻¹)
(C)
Superoxide dimutase activity (U min⁻¹ mg⁻¹ protein)
(D)
Catalase activity (μmol min⁻¹ mg⁻¹ protein)
(E)
Peroxidase activity (Unit g⁻¹ protein)
(F)
Ascorbate peroxidase activity (μmol min⁻¹ mg⁻¹ protein)
T1
T2
T3
T4

Fig. 5

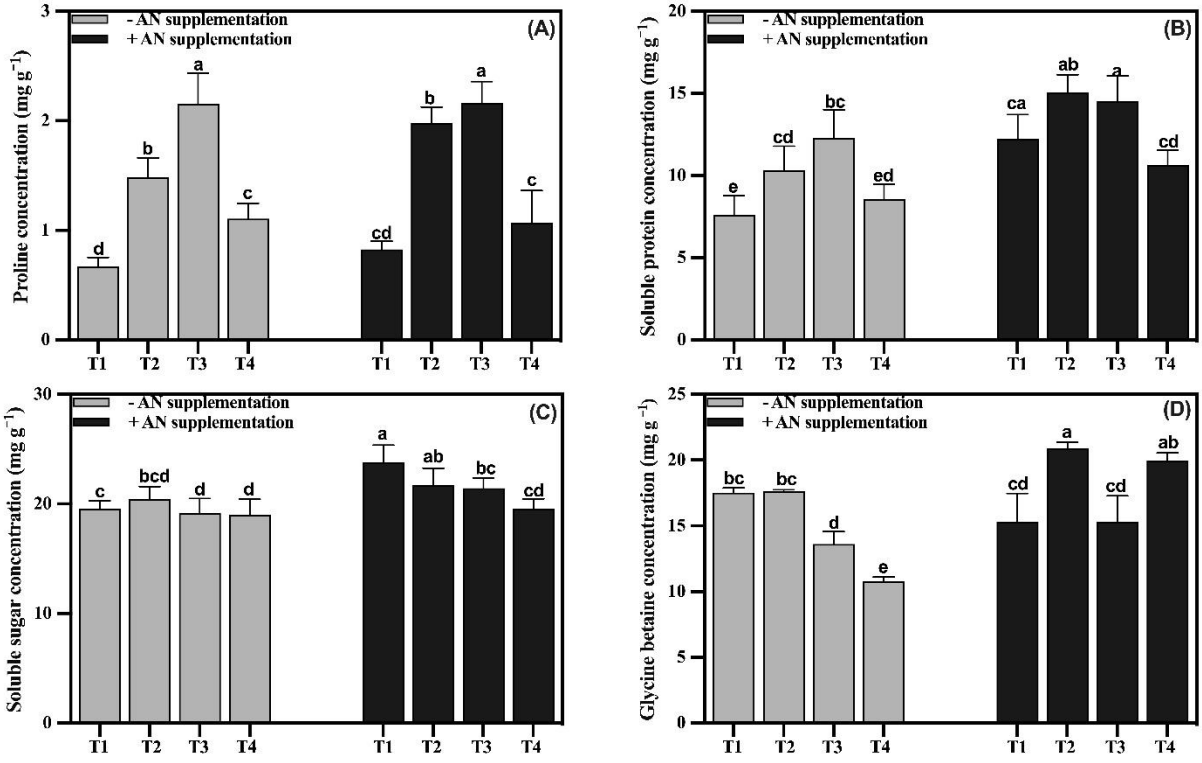

Fig. 6

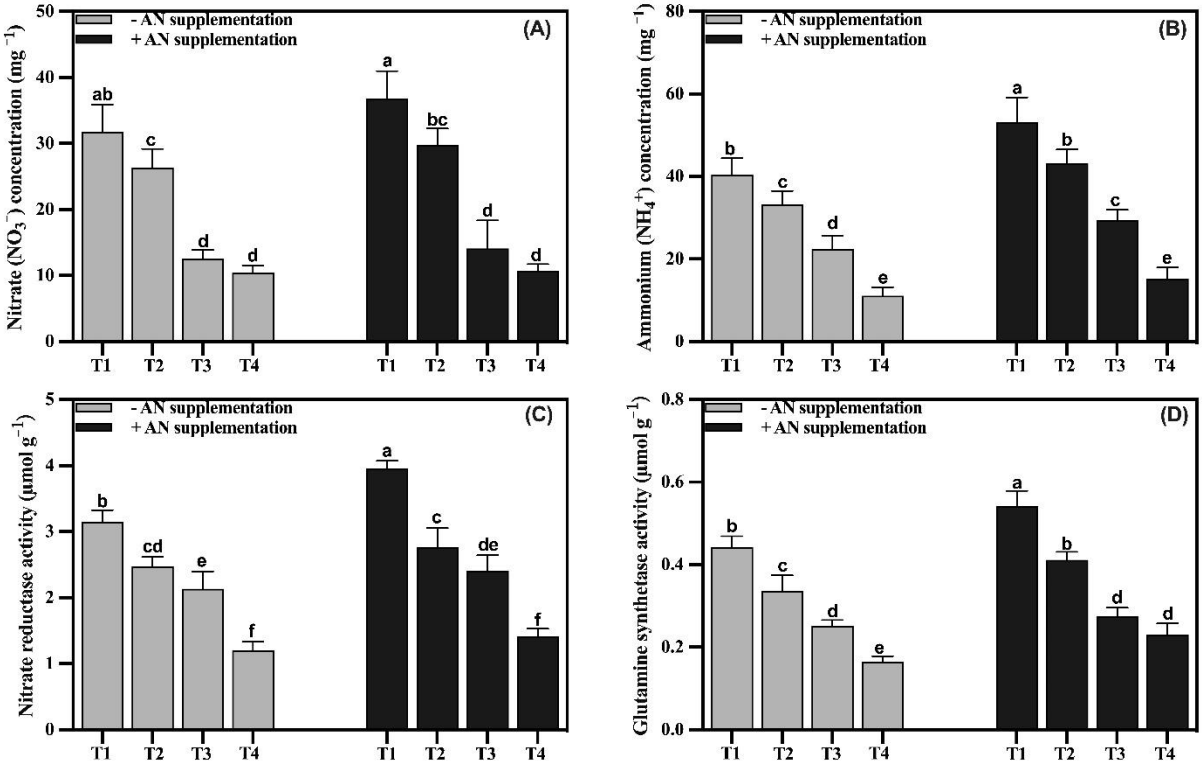

Fig. 7

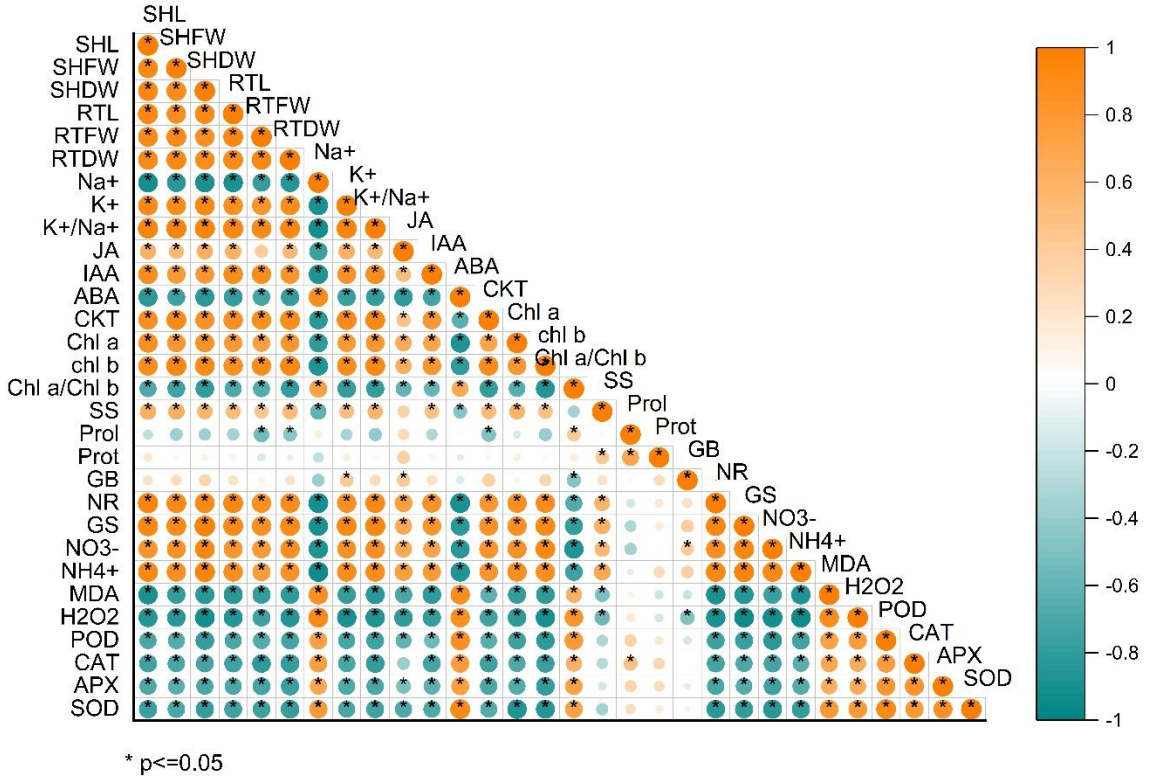

Fig. 8