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ORIGINAL PAPER

Differences in the regulation of ion imbalance in response to high Na^+ load hint at differential strategies for salt-tolerance in mungbean genotypes (*Vigna radiata L.*)

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Abstract
Development of high-yielding plant varieties resilient to environmental challenges is often hindered by the absence of genotype and growth-stage specific insights into the molecular mechanisms involved in plant survival under stress conditions. In the present study, we aimed to address this gap by analysing various physiological traits in three mungbean genotypes, viz., MGG 295, MGG 351 and LGG 460 subjected to NaCl stress (8 dS m^{-1} and 16 dS m^{-1}) during early vegetative stage. MGG 295 and MGG 351 exhibited superior salt tolerance compared to LGG 460, as evidenced by their growth performance and physiological responses, including photosynthesis, transpiration rate, membrane integrity, and reactive oxygen species (ROS) production and scavenging. Interestingly, MGG 295 showed low-ionic discrimination and non-selective uptake of Na^+ and K^+ in roots for salt tolerance. Conversely, MGG 351 exhibited low leaf and root Na^+ content, indicative of Na^+ extrusion and sequestration, similar to the salt-sensitive LGG 460. Expression of different Na^+ and K^+ transporter genes suggested *SOS1*, *SOS2*-mediated ion exclusion in LGG 460 and *NHX1*- mediated ion sequestration in LGG 460 and MGG 351. Tolerant genotypes exhibited *AKT1*-mediated K^+ uptake. Moreover, MGG 295 blocked the uptake of Cl^- suggesting an ion-wise differential strategy adopted by the plant to survive ion toxicity. These preliminary findings provide some interesting insights into the alternate approaches to salinity tolerance that are potentially less energy intensive for stress survival.

Keywords Soil salinity · Mungbean · Ion imbalance · Growth · Photosynthesis · Oxidative stress

Introduction

Salt stress is a key deterrent to agricultural productivity worldwide due to its severe impact on plant growth and development. Soil is considered saline when the electrical conductivity reaches 4 dSm^{-1} (Pirasteh-Anosheh et al. 2016). Currently, over 50% of the global population i.e. around 4.03 billion people, reside in 13 countries severely impacted by soil salinity, with projections indicating an increase to 5.02 billion by 2050 (Liu et al. 2020). An estimated 1,125 million hectares of land in these countries are affected by salinity (Hossain 2019), suggesting a staggering 50% loss of arable land due to increased salinity by 2050 (Hasanuzzaman et al. 2012). Salt stress is compounded with the induction of physiological drought and nutritional deficiencies that results in extreme toxicity hampering crop growth and metabolism. It affects plant health through various mechanisms like ionic toxicity, osmotic stress, hormonal imbalance, and oxidative stress causing cytotoxicity to various organelles and deter the functioning of several biomolecules involved in diverse biological processes in plants (Kumar et al. 2020).

Mungbean (*Vigna radiata* (L.) R. Wilczek var. *radiata)* is a highly nutritious legume widely grown in tropical and subtropical regions, particularly in Asia, Africa, and Latin America. It is a short-duration, protein-rich legume

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cultivated worldwide on approximately 7.2 million hectares, with an annual production of around 5.3 million tons (Nair and Schreinemachers 2020). India, Myanmar, and China are the leading producers, contributing significantly to global supply. It plays a crucial role in global agriculture due to its adaptability to diverse climatic conditions, short growth cycle, and high nutrition content. It serves as an important source of dietary protein for humans, fodder and forage for animals and contributes significantly to the production of edible and industrial oils (HanumanthaRao et al. 2016). In Europe also, the demand for mungbean is steadily increasing following a trend for vegan or plant-based alternatives for low-carbohydrate, high protein diet. In fact in 2021, Europe imported 45,000 tonnes of mungbean, which was approximately 40% more than a year earlier, reflecting the surge in demand (https://www.cbi.eu/market-information/grains-pulses-oilseeds/dried-mung-beans-0/market-potential). Moreover, it plays a crucial role in soil fertility improvement by fixing atmospheric nitrogen in the soil, making it an ideal rotation crop with cereals (Mehandi et al. 2019). Thus, the economic significance of mungbean is multifaceted, contributing substantially to food security, agricultural sustainability, income generation, and global trade. The global mungbean market in 2021, generated approximately USD 3,787.83 million in revenue and is projected to grow at a Compound Annual Growth Rate (CAGR) of over 3.31%, reaching nearly USD 4,757.59 million by 2028. The cumulative market growth potential from 2022 to 2028 is estimated to be around USD 30.25 billion (ICAR, 2021). Besides, mungbean is highly prized for its rich nutritional content, which includes 21–33% protein and essential micronutrients such as iron (30–60 µg/g) and zinc (20–40 µg/g). It also contains numerous health-promoting compounds like linoleic acid, tocopherols, bioactive peptides, polysaccharides, and polyphenols, all of which are beneficial in managing and preventing metabolic disorders such as diabetes, obesity, and cardiovascular diseases. Moreover, mungbean sprouts have a lower phytic acid content and higher vitamin C levels compared to other legumes like soybeans, making them an essential food source, particularly in regions like Asia and Africa, where malnutrition remains a pressing concern (Huppertz et al. 2023). Besides, an environmental impact assessment of mungbean production using the energy-water-food security nexus, reveals that mungbean is a low-impact option for protein production compared to animal products. This analysis, utilizing various nutritional units and load allocation criteria, suggests that promoting mungbean consumption can support more sustainable diets and enhance food security worldwide (Abad-González et al. 2024).

However, mungbean cultivation often occurs on marginal soils with limited inputs, rendering the crop susceptible to various biotic and abiotic stresses (Sehrawat et al. 2013). Soil salinity is a major limitation leading to significant yield losses of up to 60% at approximately 50 mM NaCl (Hasanuzzaman et al. 2012). Thus, development of climate resilient varieties resistant to environmental stress is warranted to improve and stabilize crop production. In many countries, including India and Pakistan, mungbean is commonly cultivated in soils frequently exposed to moderate to high levels of salinity (HanumanthaRao et al. 2016). To effectively utilize these salt-affected lands, it is essential to select mungbean genotypes that can tolerate salinity while maintaining substantial yields in such challenging environments. However, salinity tolerance is a complex, polygenic trait that varies depending on the genotype and growth stage (Sehrawat et al. 2013), making the development of salt-tolerant varieties a difficult task over the years. A comprehensive understanding of stress responses and molecular alterations at specific developmental stages is crucial for identifying sources of tolerance traits. Unfortunately, only a limited number of studies have compared the mechanisms of salinity stress tolerance across different mungbean genotypes. Unfortunately, only a limited number of studies have comparatively investigated the mechanisms of salinity stress tolerance in different mungbean genotypes.. Most of them were limited to screening various mungbean genotypes on the basis of growth and yield responses under salinity stress and categorising them to various levels of salt tolerance (Kumar et al. 2012; Manasa et al. 2017; Sehrawat et al. 2013; 2014; 2015; Pratiwi et al. 2021; Ahmed et al. 2024; Afzal et al. 2024; Miajy et al., 2024). As a result, there is insufficient information regarding the physiological and molecular mechanisms that enable mungbean to tolerate salinity stress. A deeper understanding of these mechanisms could significantly enhance our ability to develop a biological database of the key genes, proteins, and metabolites that lead to mungbean survival. Such data not only aids in trait prediction for improving breeding programs but also facilitates the identification of key regulatory elements for designing effective crop improvement strategies. The present study aims to fill this gap by comprehensively comparing the salt stress responses of three high-yielding mungbean genotypes viz. LGG 460, MGG 351 and MGG 295 that are commonly grown in South India, where soil salinity is rapidly becoming a prevalent issue. The study attempts to enhance our understanding of salt tolerance mechanisms in mungbean by examining genotype-specific responses at morphological, physiological, and molecular levels to osmotic and ionic toxicity induced by salinity stress. By identifying unique salt tolerance traits for each cultivar and uncovering the regulatory mechanisms involved, the research provides valuable insights to breeding programs and crop improvement strategies as well as improve our understanding of the intricacies of salt tolerance mechanisms. Ultimately, these findings will contribute to the development of more resilient agricultural systems in salt-affected regions and offer a

broader framework for exploring similar adaptations in other leguminous crops.

Materials and methods

The experimental material comprised three genotypes of mungbean (*Vigna radiata* L. Wilczek), MGG 295, LGG 460 and MGG 351. Selection of genotypes was based on available information on their high yield rate and some preliminary data about their tolerance to stress conditions (Manasa et al. 2017; Sehrawat et al. 2013; 2015; Amarapalli 2022). MGG 295 (Madhira Green Gram—295) is a widely recognized variety, particularly popular among farmers in the states of Telangana and Andhra Pradesh, India (https://pjtsau.edu.in/pdf1/8). Released in 1995, it has a crop duration of 60–65 days. It is known for its dull-seeded appearance and tolerance to Yellow Mosaic Virus (YMV). It is suitable for cultivation in all seasons. However, it has a notable susceptibility to post-harvest sprouting (Rao et al., 2023). MGG 351 (Sri Ram), released in 2016, is a high-yielding variety with an average yield of 12–14 quintals per hectare. It matures in 60–65 days and exhibits moderate tolerance to YMV. This variety is particularly suitable for cultivation during the rabi and summer seasons, as well as in rice fallows (https://www.dpd.gov.in/iii)%20Mungbean%20varieties.pdf). LGG 460 (Lam Green gram-460), released in 1997, has a crop duration of 65–70 days and is well-suited for cultivation during the Kharif, Rabi, and summer seasons (https://angrau.ac.in/downloads/CropVarities/Greengram.pdf). This variety shows moderate tolerance to YMV, has synchronous maturity, and is highly productive, yielding 15–16 quintals per hectare with a higher number of pods per cluster (http://dpd.gov.in). We used these different genotypes to explore wider genetic pool that increases the likelihood of identifying unique mechanisms and pathways that confer resilience to stress conditions. This could also potentially lead to identification of universal traits for stress tolerance that are effective across different genetic backgrounds, which can further be targeted in crop improvements to develop varieties with broad-spectrum tolerance.

Seeds of MGG 295, LGG 460 and MGG 351 were procured from Agricultural Research Station, Madhira, PJTSAU, Telangana. The following experiment was set up at the green house facility at Agri Biotech Foundation, Hyderabad during April 2022. Nursery polybags (10 kg capacity) were filled with soils (coco peat: sand and soil: vermicompost in the ratio of 1:3:1) and 10 mungbean seeds were sown in each polybag. An equal amount of water was added in each polybag before sowing in order to have sufficient moisture enabling germination of seeds. The polybags were kept in a greenhouse to avoid interference from rain, strong wind etc. Weeds were removed regularly with hands and the scheduled irrigation was manually performed at regular intervals of time. Thinning was done to five plants per bag after one week of seed germination. Salt treatment was given after the emergence and expansion of first trifoliate leaves in all the genotypes (17 days after sowing, DAS). Polybags were irrigated every alternate day with NaCl solution at 8 dSm^{-1} (~ 80 mM, ST1) and 16 dSm^{-1} (~ 160 mM, ST2) adding a total volume of 400 ml solution to each polybag per irrigation. The plants irrigated with equal volume of water were used as control (C). In mungbean cultivation, soil electrical conductivity (EC) in the field can range from 8 to 17 dSm^{-1} (Pratiwi et al 2021). Most of the mungbean cultivars tolerate salt to an extent of 9–18 dSm^{-1} during germination and behave differently to salt affliction in a genotype specific manner (HanumanthaRao et al. 2016). Thus, in this study 8 dSm^{-1} and 16 dSm^{-1} NaCl concentrations were chosen as moderate to high salt stress levels that impacts plant growth and manifests symptoms that could indicate morphological, physiological, or biochemical adaptations. The salt treatment was carried out for eight days (total 4 salinity treatments) and each treatment condition was replicated in eight polybags. Observations were recorded and samples were harvested on 25th DAS.

Growth parameters

Twenty randomly picked fresh seedlings from each treatment were collected for root and shoot length measurement using standard tapes. Similarly, the same number of seedlings per treatment were weighed for fresh weight (FW) measurement and dried at 80 °C till a constant weight is obtained and weighed for dry weight (DW) using standard methodology.

Physiological attributes of mungbean cultivars

Electrolyte leakage (EL) was measured according to the method by Wu et al. (2017). Pigment content was measured in leaf samples following the method by Arnon (1949). Leaf Photosynthetic Rate (P_n) and Transpiration rate (T_r) were measured using a portable photosynthetic system (CI-340, CID Biosciences, Washington, USA). At least twenty uniform leaf blades were analysed from each sample type by placing the upper fully expanded leaf in the leaf cuvette. IRGA was set at 0–3000 ppm CO_2 range, with the temperature of the leaf chamber set at + 25 ºC to –10 ºC. The area of the window in leaf chamber was 6.25 cm^2.

Oxidative stress markers of mungbean cultivars

Proline (Pro) was estimated according to Bates et al. (1973). The level of lipid peroxidation was determined by spectrophotometrically measuring a product of lipid peroxidation, malondialdehyde (MDA) formed after reaction with

thiobarbituric acid (TBA) (Hodges et al. 1999). Superoxide radicals ($O_2^{\cdot -}$) and Hydrogen peroxide (H_2O_2) were visually detected by staining the mungbean leaves in 6 mM nitroblue tetrazolium (NBT) in 10 mM Na-citrate buffer for 8 h and 1% 3, 3-diaminobenzidine (DAB) for 6 h under light at 25 °C (Wu et al. 2010). The quantification of DAB and NBT staining intensity was done using Image J software.

Antioxidant enzymatic activities

Fresh leaf samples (0.5 g) were homogenised in 10 mL of extraction buffer (50 mM phosphate, pH 7.8, 1.0 g polyvinyl pyrrolidone, 1 mM EDTA and 0.5% Triton X-100) at 4 ℃. The extract was centrifuged (12,000 rpm for 20 min) and the supernatant was used for protein estimation and enzyme assays. Protein content was estimated using Bradford method (Bradford 1976) using protein estimation kit (Bradford Reagent, HiMedia, Mumbai, India). Superoxide dismutase (SOD) activity was estimated using NBT method by reading the absorbance at 560 nm using a Spectrophotometer (Giannopolitis and Ries 1977). Guaiacol peroxidase (GPX) enzyme activity was measured at 470 nm using guaiacol, H_2O_2 and K–P buffer with EDTA (pH-7). Catalase (CAT) enzyme activity was determined by calculating the conversion of H_2O_2 to water spectrophotometrically at 240 nm (Chance and Maehly 1955).

Ion uptake

Dry root and leaf samples (0.1 g) were digested with nitric acid and perchloric acid (volume ratio 2:1) and was used to measure the concentrations of Na^+, K^+ and Ca^+ using a XP flame photometer (BWB Technologies, Newburg, Berks., UK). The selective K^+ uptake in leaf was calculated as described by Jones et al. (1991).

$$Selective\ K^+ uptake = \left[K^+/Na^+\right] uptake\ in\ leaf / \left[K^+/Na^+\right] uptake\ in\ roots$$

Chloride ion estimation in root and leaf sample was performed titrimetrically following Mohr's method (Skoog et al. 1996). Briefly, Cl^- extraction was performed by boiling 0.25 g dried sample in water followed by titration with 0.5 N silver nitrate. Potassium chromate was used as an end-point indicator to form red-brown precipitate of silver chromate.

Gene expression analysis

Leaf and root samples (approximately 100 mg) were used for total RNA extraction using Trizol method (RNAiso Plus, Total RNA extraction reagent, Takara). First strand synthesis was done using Prime Script RT reagent Kit (Takara, Japan). Quantitative real-time PCR was conducted on a CFX96 Real Time PCR system (Bio-Rad, USA) using PowerUP SYBR Green Master Mix-2x (Applied Biosystems, Thermo Fisher Scientific, North America, USA) following the manufacturer's instructions. The expression of different genes was calculated as log2 fold change using $\Delta\Delta C_T$ method and normalisation was done with *VrActin* as an internal reference. The sequences of primers used in this study are given in Supplementary Table 1.

Statistical analysis

All data obtained were subjected to two-way analysis of variance (ANOVA) and expressed as mean ± SE of three independent replicates. This helps to analyse the effect of two independent factor, like in our study, genotype (MGG 295, LGG 460 and MGG 351) and treatments (ST1 and ST2), on a dependent variable, such as plant physiological responses under salt stress. It reveals how different genotypes of a plant species respond to various stress treatments and whether the interaction between genotype and treatment significantly affects outcomes like growth rate, ion concentration, or stress tolerance. This analysis is particularly useful to evaluate whether certain genotypes perform better under specific treatments, or whether the response to treatment is consistent across all genotypes and is commonly used in similar studies (Sharma et al. 2015; Toderich et al. 2018). The Bonferroni correction was applied for multiple comparisons using GraphPad prism version 9.0 and the significance of difference between genotypes and stress treatment was set at $P \leq 0.05$.

Results

Salinity stress induces growth retardation in mungbean

Three different varieties of mungbean MGG 351, MGG 295 and LGG 460 treated with moderate (8 dSm^{-1}, ST1) and high salt stress (16 dSm^{-1}, ST2) showed varied responses in morphology (Fig. 1A). During vegetative growth and upon stress induction, the growth rates of different varieties was visibly observed to be different and followed the trend, MGG 295 > MGG 351 > LGG 460. Salt application affected growth and biomass of all the genotypes, although to different levels. At ST1, the shoot length in LGG 460 and MGG 351 reduced to 15% of their control values while MGG 295 showed a reduction by 8% relative to the control value. At ST2, the shoot length reduced by 34%, 23% and 15% in LGG

460, MGG 351 and MGG 295 respectively in comparison to control values. Similar trend was observed at the level of root morphology (Fig. 1B). At ST1 and ST2, LGG 460 showed a maximum reduction in the root length followed by MGG 351 and MGG 295. Biomass accumulation under salt stress showed a 33%, 8% and 11% reduction at ST1 and 48%, 31% and 19% reduction at ST2 level in LGG 460, MGG 351 and MGG 295 respectively in comparison to their respective control values. Similarly, seedling dry weight also showed that LGG 460 was impacted the most by the salt stress followed by MGG 351 and MGG 295 (Fig. 1B). Keeping in view the morphological features and percentage reduction in growth with respect to control conditions, the salt tolerance ability for the cultivars followed the trend MGG 295 > MGG 351 > LGG 460.

Salinity stress induces pigment degradation, compromised photosynthesis and gas exchange in mungbean

Salinity stress resulted in an overall reduction in the pigment content (Fig. 2A–D), photosynthesis rate (Fig. 2G) and transpiration rate (Fig. 2H) in MGG 351 and LGG 460. The concentrations of total chlorophyll (including chlorophyll a and b) and carotenoid (Car) were significantly reduced in both these varieties. For example, the total chlorophyll (Chl) in LGG 460 showed a maximum decline and was reduced to 0.41 µg gFW^{-1} at ST1 and ST2 as compared to control conditions (0.70 µg gFW^{-1}) (Fig. 2A). However, MGG 295 showed a significant increase in Chl a content from 0.28 µg gFW^{-1} to 0.32 µg gFW^{-1} at ST1 (Fig. 2B). No significant difference in the levels of Chl b and total chlorophyll was observed in MGG 295. However, Car content did not show

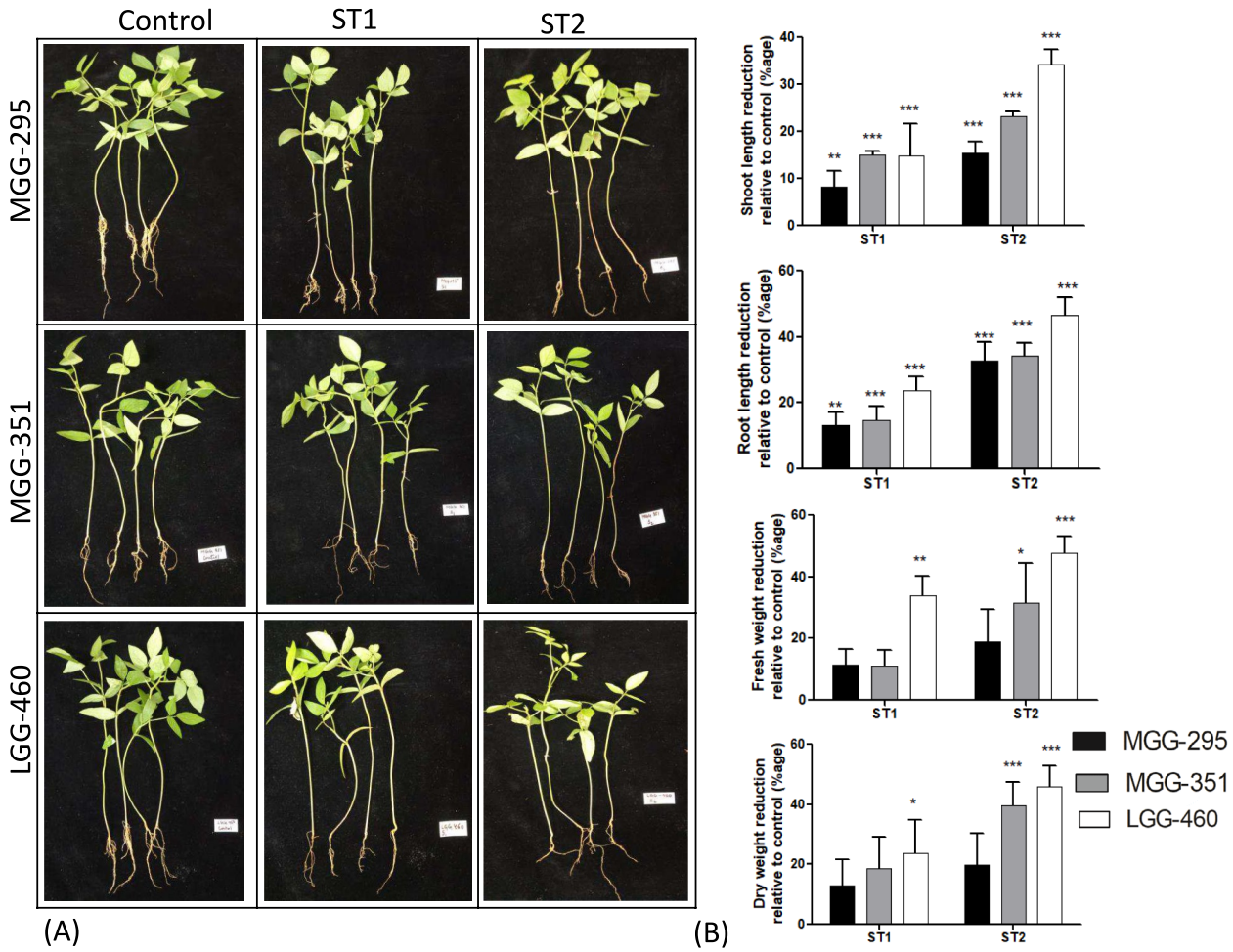

Fig. 1 **A** Morphological differences of three mungbean cultivars (MGG 295, MGG 351 and LGG 460) grown at three salinity levels (0, 8, and 16 dSm^{-1} NaCl). Plants were grown for 15 days in the pot and then irrigated with NaCl solution alternately for another eight days. **B** Graphs represents percentage reduction in shoot and root length, fresh and dry weight of three mungbean cultivars grown at two salinity (ST1, 8 dSm^{-1} NaCl) and (ST2, 16 dSm^{-1} NaCl) with respect to control conditions levels ($n = 20$). Asterisk (*) represent significant difference with respect to control (*, $P < 0.05$; **, $P < 0.001$)

such steep degradation with salt treatment. A significant decrease in Car content was seen for LGG 460 at ST1 and MGG 351 at ST2 while MGG 295 showed a significant increase in Car at ST1 (Fig. 2D). The ratio of total chl to Car chl a to chl b (Fig. 2E, F), are crucial indicators of pigment degradation under stress. We found that the total Chl/Car decreased significantly only in LGG 460 under salinity. Similarly, LGG 460 showed a significant decline in Chl a/Chl b ratio at ST2 with no significant change in ratio in other two varieties. Furthermore, salinity stress impaired the P_n of LGG 460 (11.5 umol m^{-2} s^{-1}) and MGG 351 (21 umol m^{-2} s^{-1}) at ST2 as compared to the control values in LGG 460 (27.3 umol m^{-2} s^{-1}) and MGG 351 (28 umol m^{-2} s^{-1}) while no significant difference in P_n was observed in MGG 295 under salinity stress (Fig. 2G). T_r declined only in LGG 460 under salinity with respect to its control condition while no significant difference was observed in T_r for other varieties (Fig. 2H). These results further support the relative superiority of MGG 295 for maintaining the integrity of the photosynthetic pigments and its ability to retain photosynthetic and gas exchange rate under saline condition.

Salinity stress induces membrane damage and oxidative stress in mungbean

MDA is an indicator of plasma membrane peroxidation and thus shows membrane damage. MDA content increased significantly at ST2 for all the varieties while at ST1 only LGG 460 showed a significant increase with respect to the control value. Among all the varieties, the highest accumulation of MDA was observed in LGG 460 at both ST1 (2.45 nmol gFW^{-1}) and ST2 (3.3 nmol gFW^{-1}) as compared to control conditions (1.8 nmol gFW^{-1}) (Fig. 3A). A similar trend was observed in the accumulation of proline where at

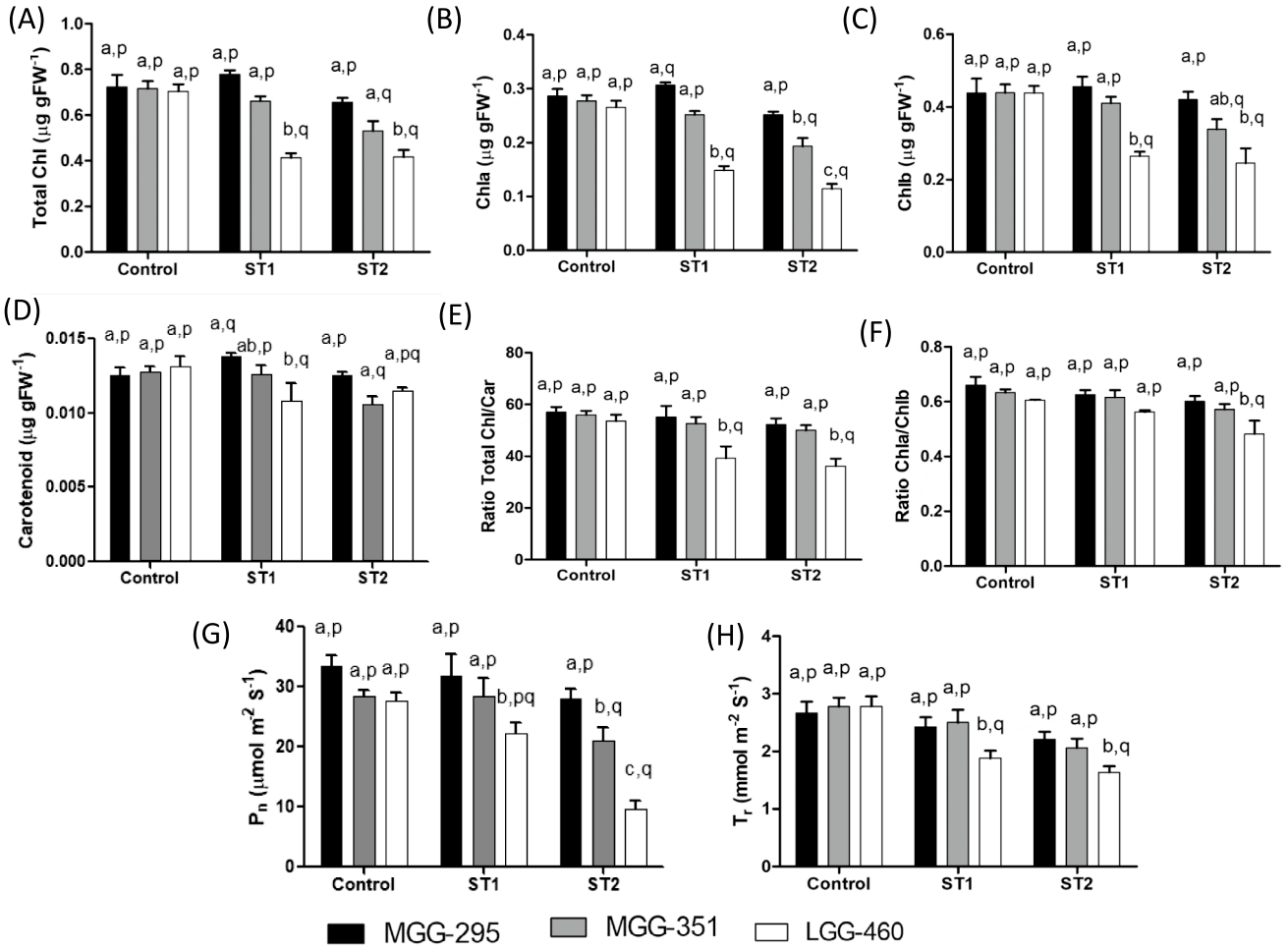

Fig. 2 Histograms show pigment content [Tolal Chl (**A**), chl a (**B**), chl b (**C**), carotenoid content (**D**)] and its degradation [Total Chl/Car (**E**), Chla/Chlb (**F**), net photosynthetic rate (P_n) (**G**) and transpiration rate (T_r) (**H**)] in three mungbean cultivars grown at three salinity levels. Values are represented as Mean ± SE (n = 3 biological replicates for pigment content, n = 20 for P_n and T_r). Data labelled by different letters (a, b and c) are significantly different among different genotypes, whereas data labelled by different letter (p,q and r) are significantly different at different salinity levels at ($P < 0.05$)

ST1 the significant enhancement in proline accumulation was observed only in LGG460 while at ST2 all the varieties showed significantly enhanced proline accumulation in comparison to their respective controls. The highest accumulation of proline was recorded in LGG 460 at ST1 (17.09 mg gFW^{-1}) and ST2 (19.97 mg gFW^{-1}) as compared to control conditions (13.3 mg gFW^{-1}) (Fig. 3B). Though an overall decrease in total soluble protein level was observed in all the varieties, significant decline was observed only in MGG 295 and LGG 460 at ST2 while MGG 351 showed no significant change (Fig. 3C).

Production of reactive oxygen species (ROS) was analysed by assessing the histochemical staining intensity of the $O_2^{\cdot -}$ (NBT staining) and H_2O_2 (DAB staining) in different varieties treated with salt (Fig. 4). The images and the relative quantification of the staining intensity also revealed that MGG 295 accumulated the least amount of $O_2^{\cdot -}$ and H_2O_2 with increasing salt concentration while LGG 460 showed the darkest staining and thus highest accumulation of ROS (Fig. 4A–C). In order to analyse the ROS scavenging potential in these varieties, we analysed the activity of three critical enzymes of antioxidant defence pathway viz, SOD, CAT and GPX (Fig. 5). Even under control condition, SOD activity was significantly higher in LGG 460 (0.11 UA mg $protein^{-1}$) as compared to MGG 351 (0.08 UA mg $protein^{-1}$) and MGG 295 (0.04 UA mg $protein^{-1}$). At ST1, a general increase in the activity of SOD was observed in all the varieties, with the highest increase in LGG 460 (0.355 UA mg $protein^{-1}$), followed by MGG 351 (0.22 UA mg $protein^{-1}$) and MGG 295 (0.09 UA mg $protein^{-1}$) in comparison to their respective controls. At ST2, a decline in SOD activity was observed in LGG 460 (0.21 UA mg $protein^{-1}$) and MGG 351 (0.14 UA mg $protein^{-1}$) though the level in activity was still higher than the control. It was interesting to see that the SOD activity continued to increase in MGG 295 even at ST2 (0.12 UA mg $protein^{-1}$) (Fig. 5A). CAT activity in MGG 295 showed a steady increase at ST1 and ST2 as compared to the control condition. However, for MGG 351, the CAT activity decreased at ST2 with no significant change in enzyme

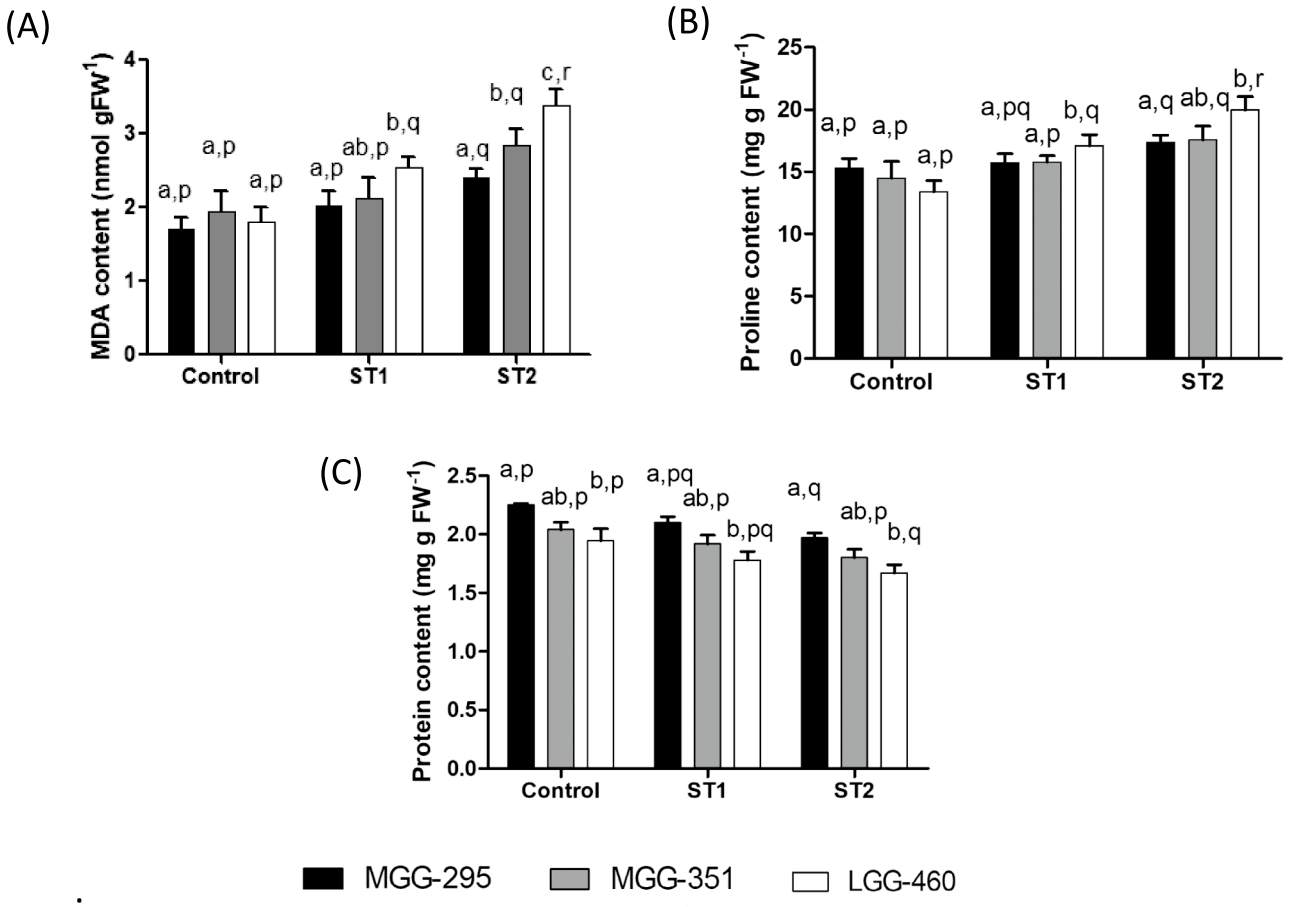

Fig. 3 Membrane damage (MDA content) (**A**), proline (**B**) and soluble protein content (**C**) in three mungbean cultivars grown at three salinity levels. Values are represented as Mean ± SE of three independent biological and three technical replications. Data labelled by different letters (a, b, and c) are significantly different among different genotypes, whereas data labelled by different letter (p, q, and r) are significantly different at different salinity levels at ($P < 0.05$)

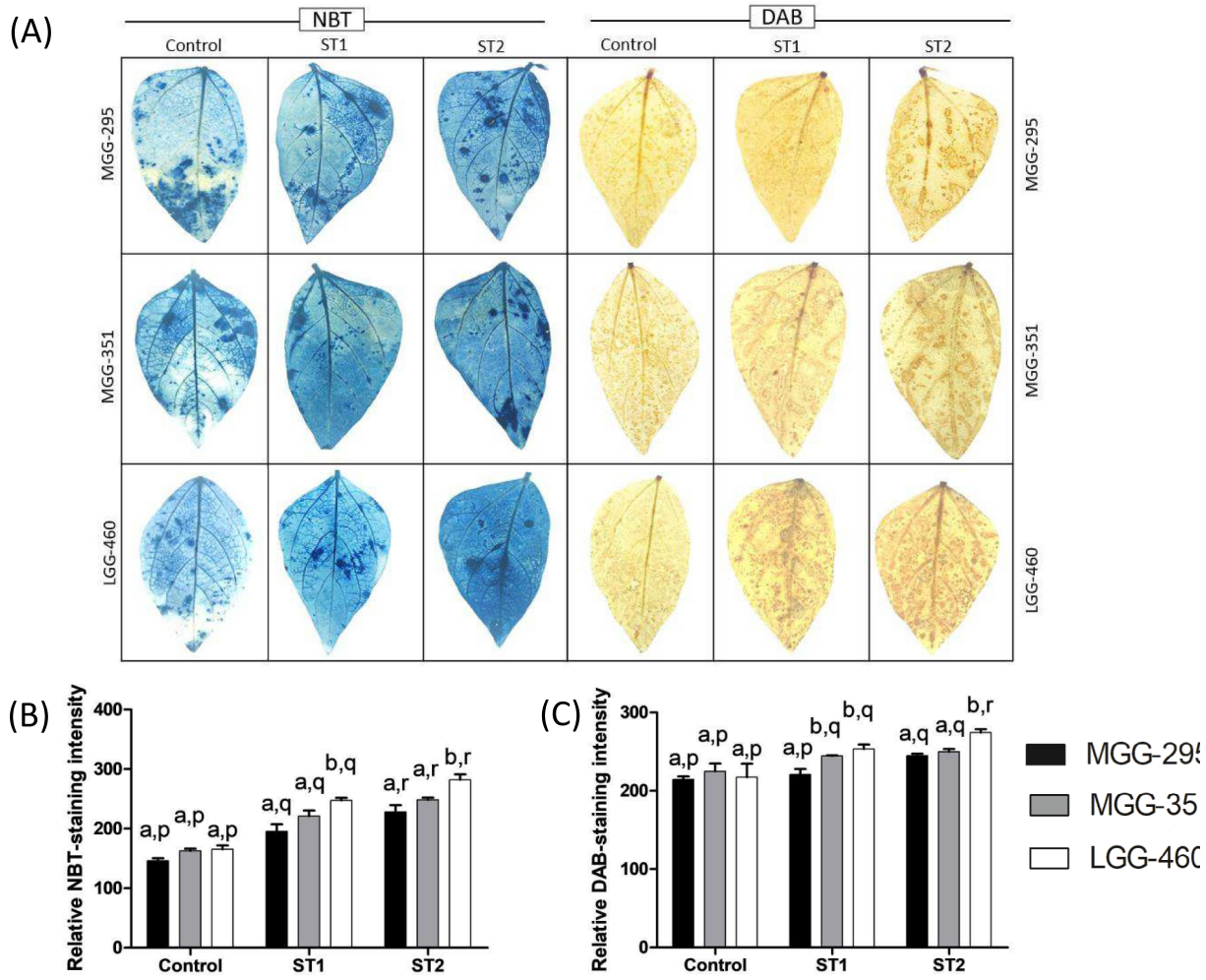

Fig. 4 Histochemical detection of ROS in leaves of mungbean genotypes inflicted with salinity stress. NBT staining of $O_2^{\bullet-}$ and DAB staining of H_2O_2 (**A**) in leaves of mungbean is represented by dark stained patches on the leaves. Quantification of the NBT (**B**) and DAB (**C**) staining was performed with Image J software and the values are represented as mean ± SD (n = 3). Data labelled by different letters (a, b, and c) are significantly different among different genotypes, whereas data labelled by different letter (p, q, and r) are significantly different at different salinity levels at ($P < 0.05$)

activity in LGG 460 (Fig. 5B). In case of GPX, a significant enhancement in the enzyme activity was observed at ST2 in LGG 460 (0.32 µmol min^{-1} mg $protein^{-1}$) and MGG 295 (0.23 µmol min^{-1} mg $protein^{-1}$) with respect to the control condition in LGG 460 (0.244 µmol min^{-1} mg $protein^{-1}$) and MGG 295 (0.191 µmol min^{-1} mg $protein^{-1}$) (Fig. 5C). No significant change in GPX activity was recorded for MGG 351.

Salinity causes ion imbalance in mungbean

Salt stress perturbed the ionic composition in roots and leaves of all the varieties. Measuring the levels of Na^+, Cl^-, and K^+ is crucial for studying ion regulation under salt stress as these ions play key roles in determining plant tolerance. Na^+ and Cl^- accumulate in plant tissues under saline conditions, causing ionic toxicity and osmotic stress, which disrupt cellular functions. K^+ on the other hand is essential for maintaining cellular homeostasis, enzyme activation, and stomatal regulation (Atta et al. 2023). Overall, an enhanced Na^+ uptake was recorded in the roots of all the varieties (Fig. 6A) with the highest Na^+ accumulated in the roots of MGG 295 at ST1 (15.03 mg gDW^{-1}) and ST2 (17.27 mg gDW^{-1}) as compared to control condition (8.9 mg gDW^{-1}). At ST1, Na^+ uptake was not significantly different among the varieties though it was significantly high in MGG 295 (17.27 mg gDW^{-1}) as compared to MGG 351 (13.1 mg gDW^{-1}) at ST2. A significant decline in K^+ uptake (Fig. 6B) in the roots of LGG 460 at ST1 (18.5 mg gDW^{-1}) and ST2 (13.9 mg gDW^{-1}) as compared to control conditions (34.5 mg gDW^{-1}) was observed while the K^+ uptake showed no significant change in LGG 295 and

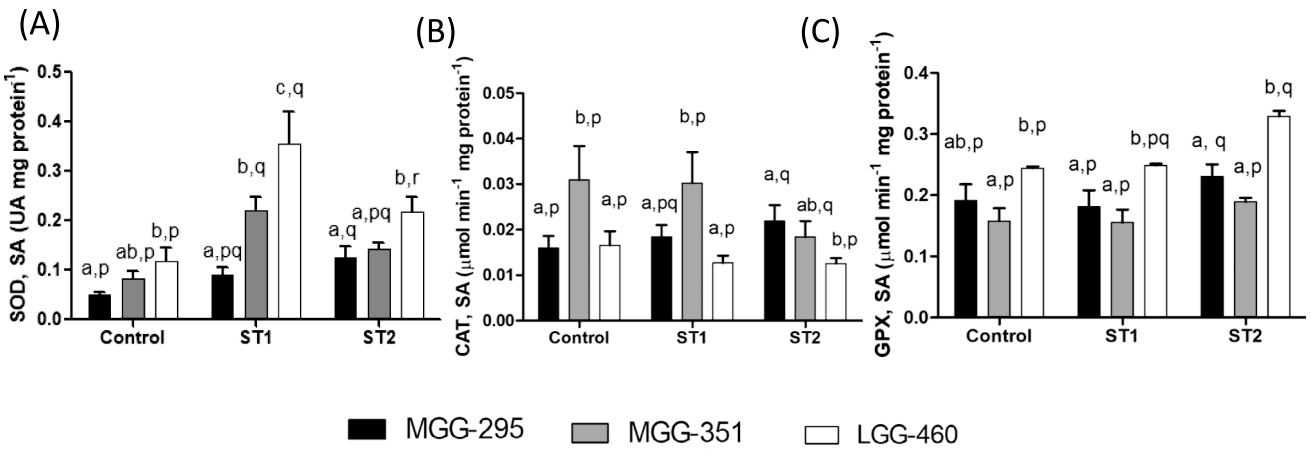

Fig. 5 Specific activity of key antioxidant enzymes (**A**) Superoxide dismutase (SOD), (**B**) Catalase (CAT), (**C**) Guaiacol peroxidase (GPX) in three mungbean genotypes grown at three salinity levels. Values are represented as Mean ± SE of three independent biological and three technical replications. Data labelled by different letters (a, b and c) are significantly different among different genotypes, whereas data labelled by different letter (p, q and r) are significantly different at different salinity levels at ($P < 0.05$)

LGG 351 under saline conditions. An overall decline in K^+/Na^+ uptake was observed in MGG 295 and LGG 460 while MGG 351 showed no significant change in the ratio. At ST2, MGG 295 recorded the highest K^+ to Na^+ uptake ratio in roots (2.02) as compared to MGG 351 (1.21) and LGG 460 (0.92) (Fig. 6C). A similar trend was seen in Na^+ translocation to leaves. Na^+ uptake saw a continuous increase in all the varieties with increasing salt concentration. The highest Na^+ uptake was observed in MGG 295 where Na^+ uptake increased from 8.53 mg gDW^{-1} under control conditions to 20.27 mg gDW^{-1} at ST1 and 25.21 mg gDW^{-1} at ST2 (Fig. 6D). Interestingly, in leaves no significant change in K^+ uptake was observed for MGG 295 and MGG 351 while LGG 460 showed a sharp decline at ST1 (93.7 mg gDW^{-1}) and ST2 (68.9 mg gDW^{-1}) as compared to control conditions (201.2 mg gDW^{-1}) (Fig. 6E). Ratio of K^+ to Na^+ to uptake also decline drastically in all the varieties though the highest ratio under salt conditions was observed in MGG 295 (Fig. 6F). The ion imbalance incurred by the salinity stress in roots and leaves helps in determining the differential selective transport (ST) capacity of the plant to transport K^+ in preference to Na^+ from root to leaf tissue. MGG 351 exhibited ionic discrimination and preferentially transported K^+ in comparison to other varieties at ST1 (Fig. 6G). Ca^{2+}, which is an important signalling molecule during stress, exhibited the lowest uptake in roots and shoots of LGG 460 as compared to other varieties at both the levels of salinity (Fig. 6H, I). Plants usually respond differentially to Cl^- ions than Na^+ and possess a separate transportation network and associated genetic machinery. Thus, we also estimated the Cl^- uptake in roots and leaves of mungbean varieties. In roots, salt stress led to a maximum uptake of Cl^- in LGG 460 at ST1 (47.07 mg gDW^{-1}) and ST2 (41.2 mg gDW^{-1}) as compared to control conditions (26.16 mg gDW^{-1}). No significant change in Cl^- was observed in MGG 295 though MGG 351 manifested an increase in Cl^- uptake at ST1(38.4 mg gDW^{-1}) and ST2 (29.1 mg gDW^{-1}) as compared to control condition (16.21 mg gDW^{-1}). A similar change in Cl^- uptake was observed in leaves where a significant uptake in Cl^- was observed only at ST2 in MGG 351 and in both the salt treatments in LGG 460 while MGG 295 showed no change in Cl^- uptake (Fig. 6J, K).

To understand the molecular genetic bases of Na^+ compartmentalisation, K^+ uptake and pH homeostasis, expression analysis of some crucial Na^+/K^+ transporter/ion channels was done. In the Na^+ transport system, the Na^+/H^+ antiporter SOS1 (Salt Overly Sensitive 1) plays a key role in expelling Na^+ from plant cells. SOS2 activates SOS1 by phosphorylating it, enhancing its function in Na^+ regulation. The Na^+/H^+ antiporter NHX1 helps sequester Na^+ into vacuoles or vesicles, reducing cytosolic Na^+ levels (Brindha et al. 2021). HKT1 maintains Na^+/K^+ homeostasis by moving Na^+ from roots to the xylem, thereby preventing excessive Na^+ accumulation in above-ground tissues. This regulation also helps alleviate the inhibition of K^+ absorption caused by high intracellular Na^+ (Laurie et al. 2002; Davenport et al. 2007; Song et al. 2024). AKT ensures efficient K^+ absorption, even when Na^+ concentrations are elevated (Nieves-Cordones et al. 2010). Vacuolar ATPases (V-ATPases) actively pump protons (H^+) into the vacuole, creating an electrochemical gradient that drives the transport of various ions, including Na^+ and K^+, across the vacuolar membrane via antiporters such as NHX (Seidel 2022) These genes are often studied to evaluate the ion homeostasis under salt stress in various plant species including legumes like soyabean (Sun et al. 2019; Wang

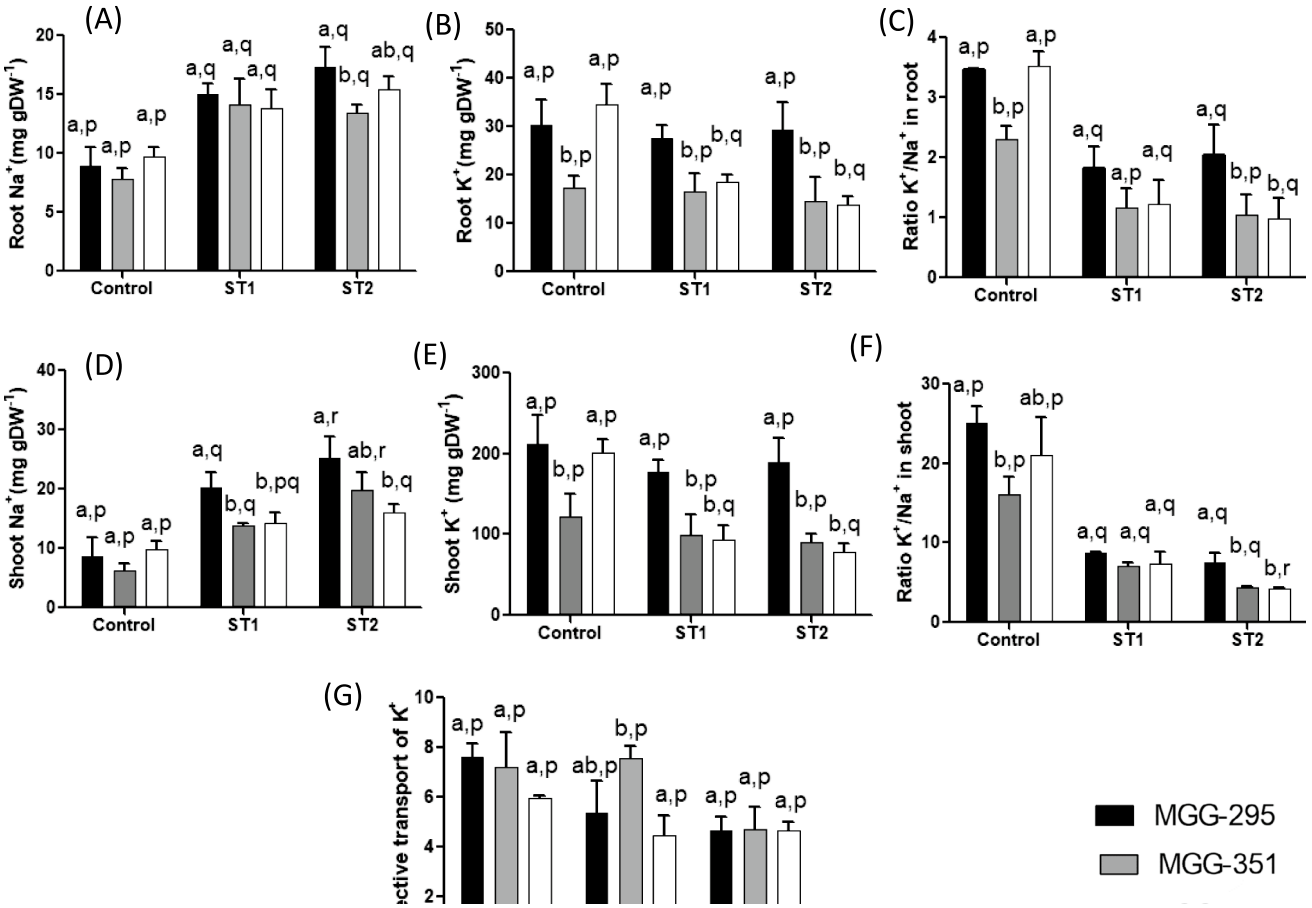

Fig. 6 (**A**, **D**) Na^+, (**B**, **E**) K^+ contents, (**C**, **F**) K^+/Na^+ ratio, (**G**) selective K^+ uptake, (**H**, **I**) Ca^{2+} and (**J**, **K**) Cl^- uptake in root and leaves respectively, in the three mungbean genotypes grown at three salinity levels. Values are represented as Mean ± SE (n = 5). Data labelled by different letters (a, b and c) are significantly different among different genotypes, whereas data labelled by different letter (p, q and r) are significantly different at different salinity levels at ($P < 0.05$)

et al. 2021) and ground nut (Asif et al. 2011). In this study, we also used these genes as markers for ion exclusion and sequestration. In leaves, expression of plasma membrane sodium transporter gene *SOS1* was induced at both levels of salinity in LGG 460 (Fig. 7A). Interestingly, MGG 295 and LGG 351 showed a significant downregulation of *SOS1* at ST1. However, in roots (Fig. 7B), *SOS1* was downregulated to more than fourfold in LGG 460 while a significantly induced expression was observed in MGG 351 at ST2. Similarly, *SOS2* was induced more than fivefold at ST2 in LGG 460 in leaves while in root samples it was decreased to more than sixfold (Fig. 7C, D). A significant decline in the expression was observed in *SOS2* at both the levels of salinity in MGG 295 and at ST2 in MGG 351 while a sevenfold induced expression was observed in MGG 351 at ST1 in root samples (Fig. 7C, D).

Tonoplast-localized K^+, Na^+/H^+ antiporter *NHX1* transporter was upregulated significantly at ST1 and ST2 in MGG 351 and ST1 at LGG 460 in leaves while no significant change in expression occurred in root samples (Fig. 7E, F). AKT1 is an important member of the K^+ transporter family (KT). Interestingly, *AKT1* expression in leaves was down-regulated in LGG 460 (more than sixfold) while it was induced to more than fivefold in both MGG 295 and MGG 351 at both the salinity levels. However, in roots, a significant change in expression was observed only for MGG 295 at ST1 where threefold decline was recorded (Fig. 7G, H). Another non-specific sodium/potassium transporter *HKT1* showed induced expression in leaves in MGG 351 (ST2) while a downregulated expression was seen in both levels of salinity in MGG 295. In roots, the significant change in expression was observed only for MGG 295 (ST1) and MGG 351 (ST1) where a significant increase and decrease in expression was observed respectively (Fig. 7I, J). We also looked at the expression of the pH-related transporter proton pumps and found that the expression of vacuolar *ATPase-A* (Fig. 7K, L) was induced significantly at ST2 in MGG 351 and LGG 460 with more than tenfold increase in MGG 351 at ST2. MGG 295 on the other hand, showed a downregulation of *ATPase-A* to more than eightfold at

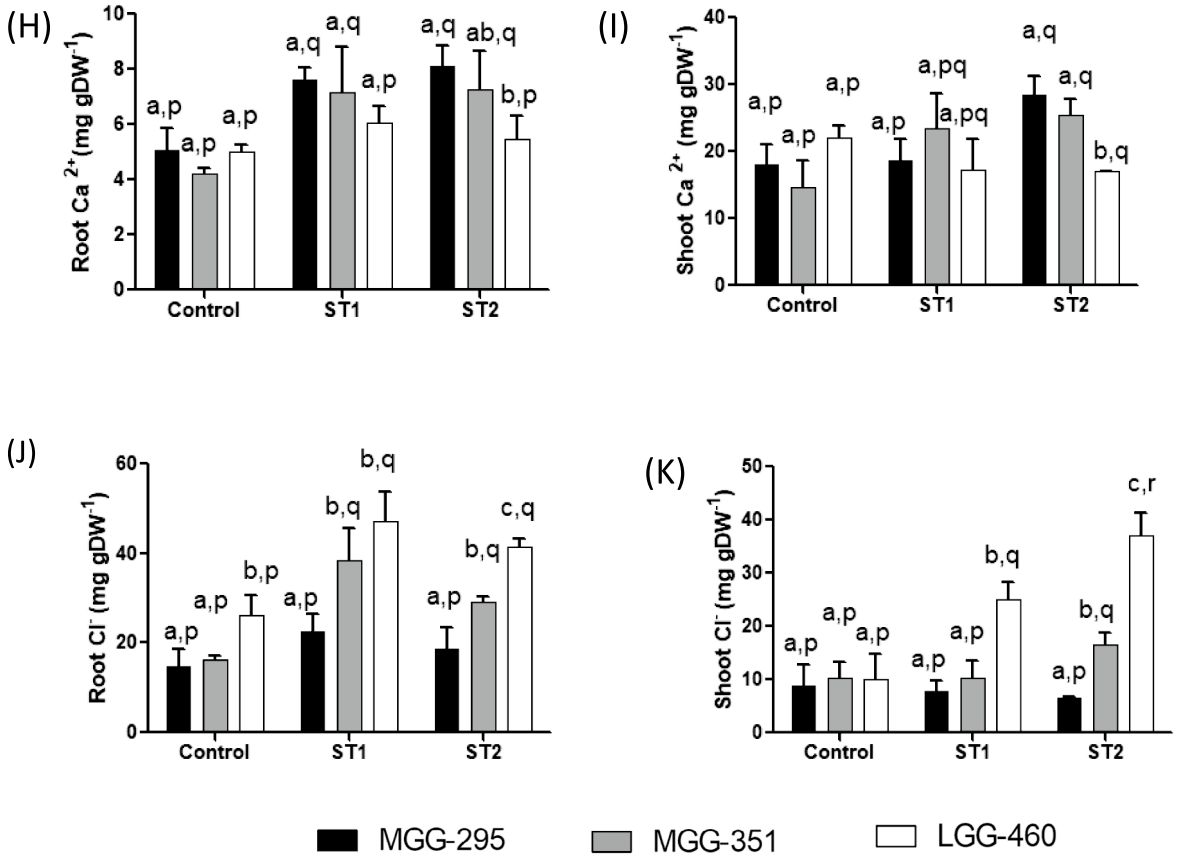

Fig. 6 (continued)

ST1. In roots however, its expression was induced many folds in MGG 295 at ST2 while a decline in the expression was observed at ST1 and in MGG 351 at ST2. *ATPase-D* expression (Fig. 7M, N) was reduced significantly in MGG 295 at ST1 while it induced significantly in leaves sample of MGG 295 at ST2 with a 12-fold enhancement. We also looked at the expression of chloride transport gene *CLC-b2* and *CLC-c2*. We found that the expression of *CLC-b2* declined significantly in leaves of MGG 295 while a significant increase was recorded for LGG 460. The expression of *CLC-b2* increased significantly to more than fourfold in LGG 460 roots (Fig. 7O, P). A similar trend was observed in the expression of *CLC-c2* in leaves where a significant decline in its expression was observed only in MGG 351 and MGG 295 at both the salinity levels. In roots on the other hand, LGG 460 showed an enhanced expression at ST2 (Fig. 7Q, R).

Correlation analysis

Pearson's correlation analysis was conducted to assess the differential correlations observed between plant growth, physiology, biochemical attributes, and ion homeostasis for the two most contrasting varieties, LGG 460 and MGG 295 (refer to Fig. 8). In the correlation matrix, insignificant correlations ($P > 0.05$) have been denoted as blank. In LGG 460, root and leaf Na^+ content exhibits a strong negative correlation with shoot length, fresh weight, chl a, and chl b, while root Na^+ content is negatively correlated with P_n, T_r, Chl a and chl b. These findings suggest that the accumulation of Na^+ ions is associated with reduced biomass, photosynthesis, and transpiration in LGG 460. Contrarily, in MGG 295, Na^+ content in the roots shows a strong positive correlation with K^+ and Ca^{2+} levels in the roots. This indicates that Na^+ uptake does not hinder the uptake of K^+ and Ca^{2+}. Na^+ content in leaves and roots also show a positive correlation with Car content in MGG 295.

Discussion

This study aims to thoroughly explore the physiological and molecular mechanisms underlying salt stress tolerance across three mungbean cultivars. It delves into three important attributes of salinity stress: (1) osmotic stress

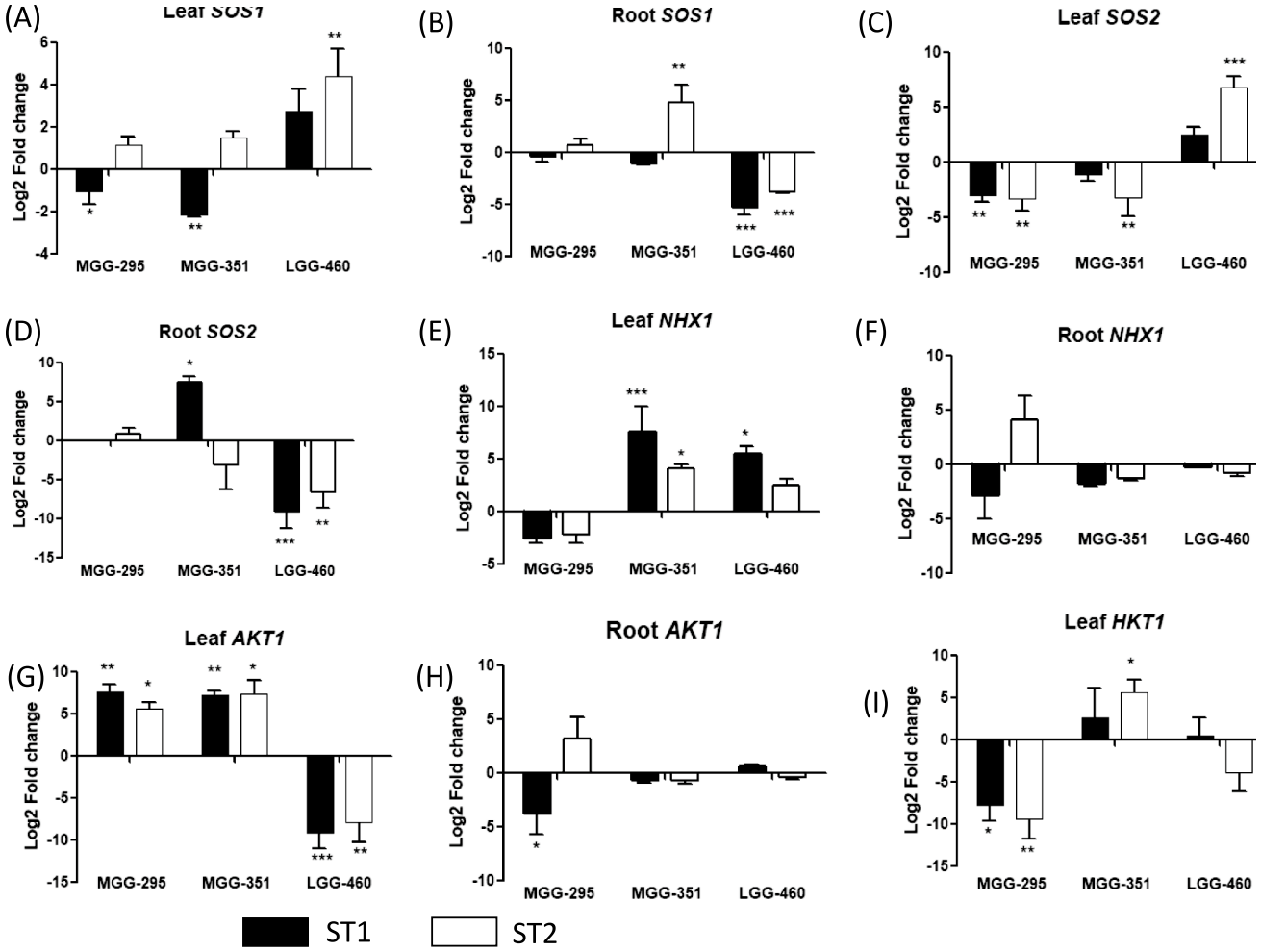

Fig. 7 Bars represent expression profile (relative expression in terms of Log_2 fold change with respect to control value) of different Na^+/H^+ transporters, viz., *SOS1* (**A**, **B**), *SOS2* (**C**, **D**) and *NHX1* (**E**, **F**); different K^+/Na^+ transporters, viz., *AKT1* (**G**, **H**), *HKT1* (**I**, **J**); and vacuolar H^+ pumps, viz., *V-ATPase a* (**K**, **I**) and *V-ATPase d* (**M**, **N**) and chloride uptake and transport gene *CLC-b2* (**O**, **P**) and *CLC-c2* (**Q**, **R**) in roots and leaves of mungbean genotypes subjected to salinity stress. The asterisks *,**,*** represent significant change at $0 < 0.05$, $P < 0.01$ and $P < 0.001$ in comparison to respective control. The values presented are the mean ± SE of three independent biological and three technical replications. Black bars represent salinity level ST1 and white bar represent ST2

induced by elevated soil salinity levels, (2) oxidative stress due to salt-induced injury, and (3) ion toxicity within the cellular environment. Figure 9 represents a snapshot of the metabolic rearrangements that have been recorded in the present study in three contrasting genotypes for salinity tolerance, MGG 295 and LGG 460.

Osmotic adjustment and redox regulation in response to salt stress

Osmotic stress is the primary phase of salinity stress which involves a decrease in hydraulic conductance that impedes water and solute uptake by plants. It leads to water and nutrient deficit that retards plant growth and development (Munns and Tester 2008). Salinity stress resulted in the reduced seedling growth and biomass as observed in other leguminous crops like soyabean (Chen et al. 2024), common bean (Raggi et al. 2024) and groundnut (Joshi et al., 2024). Of all the three genotypes, MGG 295 showed better growth and biomass under control and saline conditions reflecting an innate ability to withstand salinity. LGG 460 displayed all the traits associated with salt stress injury including fewer photosynthesis pigments, lower photosynthesis rate, lower transpiration rate (Ben Ahmed et al. 2011; Wani et al. 2016) that result in observed reduction in length and biomass of seedlings. Interestingly, a slight increase in chl content and Car was observed in MGG 295 at ST1 that was similar to salinity induced increase in chlorophyll content observed in other leguminous crops like common bean (García et al. 2024) and soyabean (Wang et al. 2001). In fact, similar to MGG 295, in soyabean chl content increased up to 10 dsm^{-1} of NaCl stress (Wang et al. 2001). This could indicate the

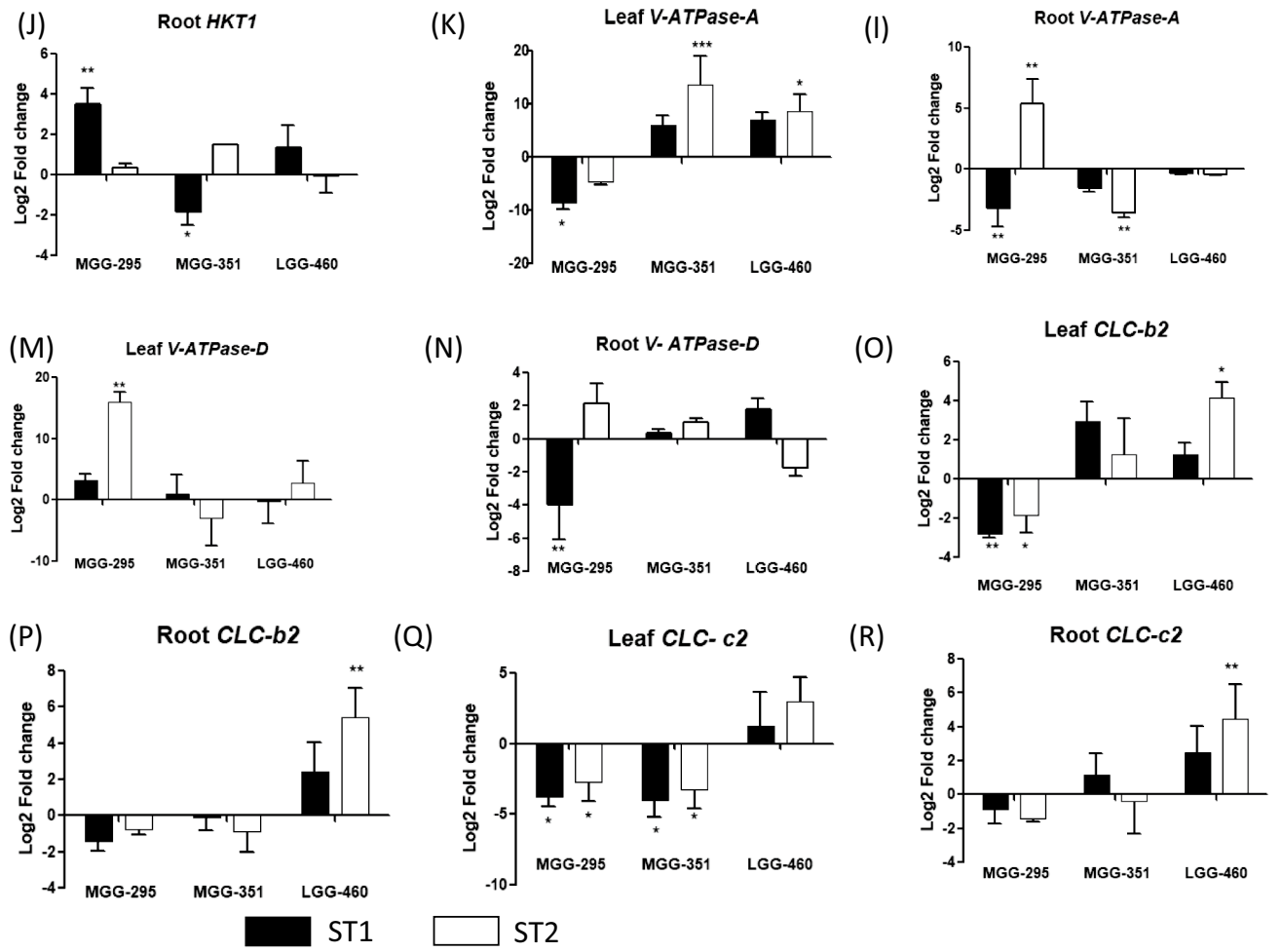

Fig. 7 (continued)

capacity of the tolerant genotype to retain the integrity of pigment upto a level of salt exposure at which sensitive genotype underwent chlorophyll degradation. Moreover, Car are involved in the transcriptional modulation of several genes responsive to ROS and thus, are retained as a protective mechanism against stress conditions (Shumbe et al. 2014; Qiu et al. 2017). Furthermore, proline was accumulated to very high levels in LGG 460 both at ST1 and ST2 as compared to other varieties. Proline accumulation is usually associated with increased stress tolerance and a common response to salinity stress in plants including several legumes like common bean (Gupta and Pandey 2020; Dawood et al. 2022) and soyabean (Noor et al. 2024). Nevertheless, several plant species sensitive to stress exhibit very high accumulation of proline under stress conditions as observed in wheat, alfa alfa, soyabean, oil palm, Amaranth (Poustini et al. 2007; Wang and Han 2009; Noor et al. 2024; Li et al. 2019; Sarker and Oba 2020) indicating that proline accumulation may not necessarily indicate plant resistance. Instead, it could also be a consequence of stress injury rather than the cause of stress tolerance (Spormann et al. 2023). Genetic studies also show that disturbed proline homeostasis cause reduced proline catabolism that contribute to elevated levels of proline under stress (Kavi Kishore and Sreenivasulu 2014).

Salinity induced the highest $O_2\cdot^-$ and H_2O_2 production in LGG 460 and least in MGG 295. Concomitantly, enzyme activities of key antioxidative defence enzymes were also enhanced under salt stress. The enzyme activity of SOD and GPX was elevated in LGG 460 at higher level than that of MGG 295 at ST1. This could be related to the lesser $O_2\cdot^-$ generation in MGG 295 due to lower membrane damage (lower MDA content) and efficient photosynthesis. Moreover, higher Car content in MGG 295 provide additional protection as Car is involved in scavenging the lipid peroxy-radicals and inhibit superoxide generation (Farooq et al. 2009). Thus, a lower SOD activity is sufficient for dismutation of generated $O_2\cdot^-$ in MGG 295. Similar instances of increase in SOD and other antioxidant enzymes are also seen in sensitive cultivars of other crops like rice (Deus et al. 2015; Vaidyanathan et al. 2003;

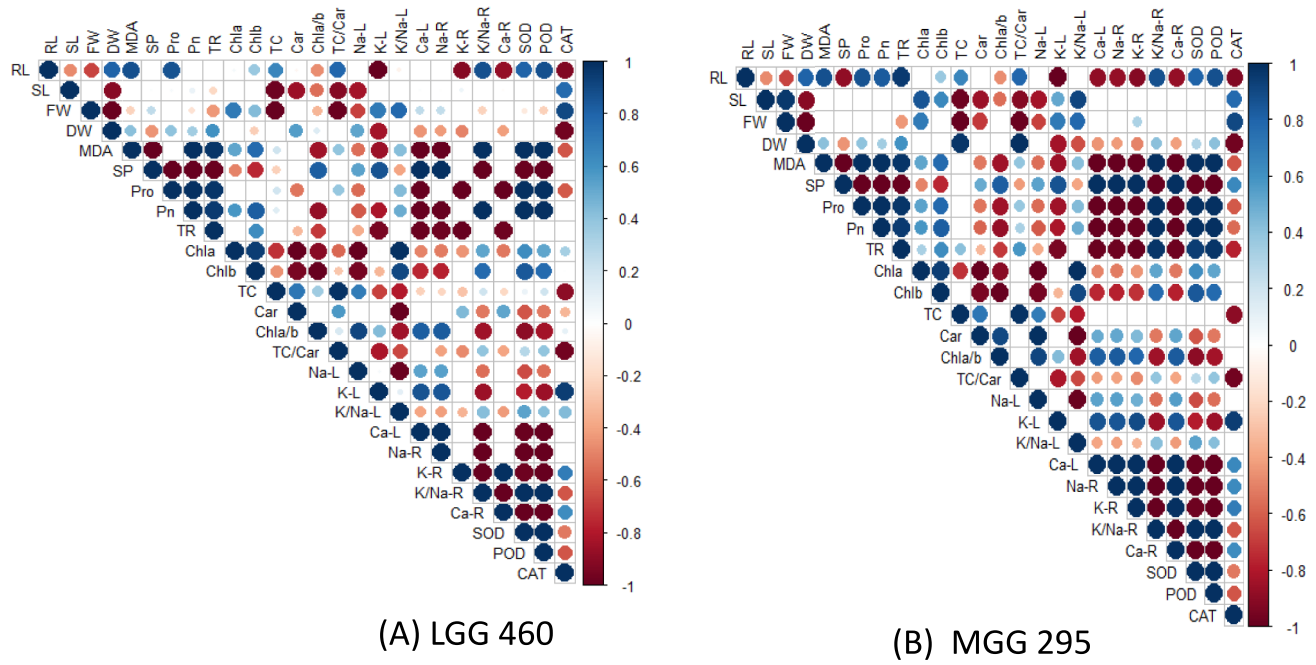

Fig. 8 Correlation analysis ($P < 0.05$) between various measured attributes of **A** LGG 460 and **B** MGG 295. The abbreviations are as follows: DW (dry weight), P_n (net photosynthesis rate), T_r (transpiration rate), Chl a (chlorophyll a), Chl b (chlorophyll b), T. Chl (total chlorophyll), Car (carotenoid content), SOD (superoxidase activity), CAT (catalase activity), SP (soluble protein content), Pro (proline content), MDA (malondialdehyde content), EL (electrolyte leakage), Na–R (sodium concentration in roots), Na–L (sodium concentration in leaves), K–L (potassium concentration in leaves), K–R (potassium concentration in roots), Na–T (Na^+ translocation), K–T (K^+ translocation), K/Na ratio (K^+/Na^+ ratio), Na uptake (Na^+ uptake). The value on scale bar between −1 and 1 are representative of degree of correlation, with a value of −1 meaning a total negative correlation, 0 being no correlation, and + 1 meaning a total positive correlation

Sharma et al. 2013) and several other legumes like common bean (Gupta and Pandey 2020; Taïbi et al. 2021; Dawood et al. 2022) and soyabean (Qian et al. 2024). Notably, the initially heightened SOD activity observed in LGG 460 and MGG 351 during ST1 decreased by ST2, likely due to enzyme inhibition caused by the elevated production of ROS. Conversely, MGG 295 exhibited a consistent increase in SOD activity showing the retention of enzyme functionality at higher salt treatment. Additionally, LGG 460 displayed higher GPX activity but lower CAT activity compared to MGG 295. H_2O_2 is decomposed by different peroxidases such as GPX found mainly in apoplast and vacuole (Takabe et al. 2001) while CAT mostly decompose H_2O_2 generated during photorespiration in the peroxisome (Dat et al. 2000). The variation in the activity level of the enzymes could be hinting towards differential H_2O_2 load in cellular compartments (peroxisomes, apoplast and vacuole) in LGG 460 and MGG 295.

Genotypic difference in ion homeostasis under salt stress

The regulation of intracellular Na^+ homeostasis to maintain high cytosolic K^+/Na^+ ratio has become a pivotal salinity tolerance trait (Munns and Tester 2008). In several legumes, higher concentrations of Na^+ and Cl^- ions in leaves imbalanced the cytoplasmic ion levels and metabolic pathways, eventually hindering the photosynthesis process and productivity as observed in soybean (Cai et al. 2022), groundnut (Li et al. 2024) and common bean (Dawood et al. 2022). Interestingly, at a low salinity level (ST1), there was no significant difference in Na^+ influx in the roots of salt-tolerant and sensitive lines. A similar response was also observed under lower salinity exposure in non-halophyte like barley (Shabala et al. 2010) and wheat (Davenport et al. 1997) indicating that regulation of Na^+ uptake might have a relatively minor role in the overall stress tolerance at lower salt exposure. However, at ST2, MGG 295 displayed the highest accumulation of Na^+ ion in both roots and leaves as compared to other genotypes. Similar response was seen in salt-tolerant wheat (Wu et al. 2016), barley (Shabala et al. 2010) and lettuce (Bartha et al. 2015) that accumulated higher cellular Na^+ in comparison to their sensitive counterparts suggesting that inverse relationship between tissue Na^+ concentration and salinity tolerance is not universal. This behaviour is also reminiscent of halophytes (Katschnig et al. 2015) as well as salt-tolerant non-halophyte that accumulate Na^+ and Cl^- in the root and leaves cells at concentration similar to the external solution, resulting in an energy-efficient

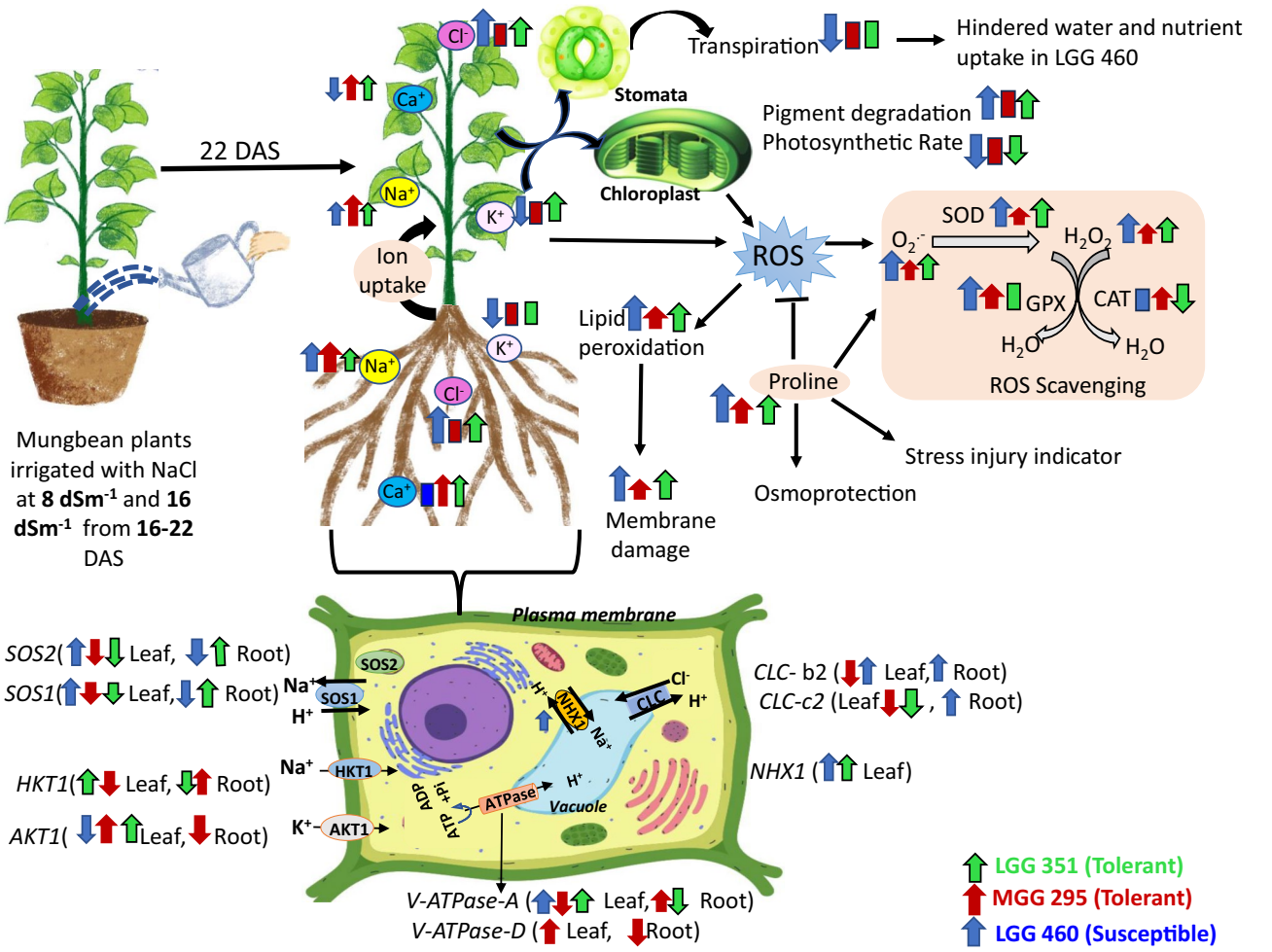

Fig. 9 Schematic representation of differential salt stress responses at the level of growth, physiology, osmotic balance and regulation of genes governing ion balance in three genotypes, LGG 460, MGG 351 and MGG 295. Mungbean seeds were germinated in pots and salt stress was inflicted after the emergence and expansion of first trifoliate leaves (16 DAS). Plants were irrigated with NaCl solution at 8 and 16 dSm^{-1} every alternate day. Various parameters were studied and depicted in the figure. Red, blue and green arrow represent the studied parameters in MGG 295, LGG 460 and MGG351 respectively. Directionality of arrows represent decrease () or increase () in the values. Height of the arrow represent the intensity of the response. Coloured solid bars represent no change under salinity stress. The figure represents the differential ion uptake in roots and leaves of all genotypes. The figure also depicts the differential regulation of genes involved in ion uptake and transport. Salinity stress leads to increased pigment degradation and reduced transpiration and photosynthetic rate in LGG 460. Salinity-induced osmotic stress and ionic stress trigger the overproduction of ROS which react with membrane phospholipids leading to lipid peroxidation. LGG 460 recorded a highest production of ROS and membrane damage. Antioxidant enzymes were activated to scavenge the ROS. Increased production of proline is a usual response under salt stress. It provides osmoprotection and controls ROS production through direct scavenging of radicals and by increasing expression or activity of antioxidant enzymes. However, higher proline production in LGG 460 could also indicate stress injury

osmotic adjustment (Shabala 2013; Munns and Gilliham, 2013). Infact, MGG 295 also showed a low increase in proline content under saline stress indicating the potential use of high Na^+ as osmoticum. Additionally, Na^+ can also sometimes act as a "non-essential" or "functional" nutrient (Maathuis 2014). Moderate levels of Na^+ concentrations can serve as a beneficial factor stimulating growth, as observed in various salt-sensitive plants like rice (Horie et al. 2007), sugar beet (Katerji et al. 2003) and maize (Rehnama et al. 2010) possibly by replacing vacuolar K^+ with Na^+ leaving more K^+ free for cytosol (Wu et al. 2018). The least Na^+ uptake in roots was observed in MGG 351, suggesting that salt stress adaptation in MGG 351 operates through Na^+ exclusion. Although, LGG 460 took up high Na^+ ion in roots, it restricted the upward transport of Na^+ from root to leaves probably due to superior xylem unloading or vacuolar sequestration of Na^+ (Wu et al. 2018). These results are supported by the expression of Na^+/H^+ antiporter in the plasma membrane (*SOS1* and its associated *SOS2)* and tonoplast (*NHX1)* that control Na^+ export and vacuolar sequestration

respectively (Brindha et al. 2021). A significantly downregulated expression of *SOS1* and *SOS2* genes in LGG 460 (roots) and MGG 295 (leaves) suggest a suspension of Na^+ exclusion mechanism. However, in leaves, LGG 460 appeared to employ Na^+ exclusion (*SOS1* and *SOS2*) and sequestration (*NHX1*) as revealed by significant upregulation of these genes. On the other hand, the lower root Na^+ concentration in MGG 351 as compared to other varieties could be due to the enhanced exclusion by significantly upregulated *SOS1*. Furthermore, MGG 351 and LGG 460 perform enhanced compartmentalisation as shown by significantly upregulated *NHX1,* to mitigate Na^+ toxicity in leaves. Na^+ exclusion in MGG 351 could also possibly occur through HKT1-type transporters that regulate Na^+ uptake in roots (Laurie et al. 2002) and Na^+ transport from xylem vessels to phloem for shoot-to-root Na^+ recirculation (Davenport et al. 2007; Song et al. 2024). A similar reciprocal regulation of expression of *HKT1* with significantly reduced expression in roots and induced expression in leaves as found in MGG 351, is also seen in case of *HKT*-mediated microbe induced-enhanced Na^+ tolerance in Arabidopsis (Zhang et al. 2008). Moreover, NHX1 activity relies on an H^+ gradient across the tonoplast maintained by vacuolar H^+−ATPases (V-ATPase-A and D) (Silva and Geros 2009) and thus increased activity of Na^+ /H^+ antiporters should coincide with the increased activity of tonoplast H^+-ATPases.Significantly enhanced expression of *V-ATPase-A* in LGG 460 and MGG 395 leaves corroborated with enhanced sequestration through *NHX1*. The significantly enhanced expression of *V-ATPase-D* in leaves in MGG 295 could be involved in providing tolerance to oxidative stress as seen in Arabidopsis (Feng et al. 2020).

Salt stress generally induces K^+ efflux from tissue and many plants manifest a strong positive correlation between the ability to retain high root K^+ and salinity stress tolerance (Wu et al. 2018; Shabala and Pottosin 2014). MGG 295 showed the highest accumulation of K^+ ion in roots and leaves, while the other two genotypes underwent a high K^+ efflux from both root and leaves. Several non-halophytes like barley and wheat are known to retain large quantities of K^+ for salt tolerance (Chen et al. 2007b; Cuin et al. 2008). In fact, K^+ contributes to about 35 to 50% to cell osmotic potential, besides its other roles including enzyme activation, pH homeostasis, antioxidative defence (Shabala and Pottosin 2014). The significantly elevated expression of the K^+ transporter *AKT* in leaves of MGG 295 supports higher K^+ uptake and transport under saline conditions (Nieves-Cordones et al. 2010), although its significantly high expression in MGG 351 did not translate into high K^+ uptake. Interestingly, MGG 351 uniquely adopts selective transport of K^+ in leaves as compared to roots at ST1. This selectivity in K^+ uptake sheds light on the strategy of plant to prioritise the maintenance of K^+ related functions in leaves to withstand stress (Shabala and Pottosin 2014).

Salt tolerance in many species is related to control of Cl^- transport and exclusion and different varieties could show varied response to Cl^- ion toxicity (Luo et al. 2005). In fact, salt stress related traits have been associated with Na^+ and/or Cl^- toxicity in soybean (Luo et al. 2005), mungbean (Salim and Pitman 1983), cowpea (Praxedes et al. 2009), and common bean (Ashraf and Bashir 2003). For instance, in soybean salt-induced damage is related to Cl^- content in the aerial part (Pantalone et al. 1997). The successful exclusion of shoot Cl^- is important for the salt tolerance of cultivated soyabean (*Glycine max*) which is more sensitive to Cl^- than Na^+ in comparison to the wild variety *G.soja* (Luo et al. 2005). In a recent report, evaluation of growth, photosynthesis, and tissue ion concentrations in single genotype each of soyabean, cowpea, mungbean and common bean exposed to NaCl, Na^+ (without Cl^-), and Cl^- (without Na^+) revealed that salt sensitivity is predominantly determined by Na^+ toxicity in soybean, Cl^- toxicity in mungbean, and both Na^+ and Cl^- toxicity in cowpea and common bean (Le et al. 2021). In our study also, MGG 295 adopts the classical approach of Cl^- exclusion to limit its uptake in roots. This is evident from the no significant change in Cl^- concentrations observed in both roots and leaves, which aligns with the significantly decreased expression of chloride transport genes, *ClC-b2* and *ClC-c2*, under salinity stress. A recent study in contrasting genotypes in salt tolerance in mungbean also identifies Cl^- exclusion and maintenance of high K^+ in root and leaf mesophyll as responsible factors for the salt tolerance trait (Iqbal et al. 2024). In comparison, MGG 351 exhibits higher Cl^- ion uptake in roots but effectively impedes its translocation to leaves, at least at ST1, indicating ion compartmentalization. In LGG 460, a significant uptake of Cl^- is observed in roots and leaves which could attribute to the known toxicity symptoms of Cl^- such as enhanced membrane damage and ROS production (Khare et al. 2015), reduced photosynthesis and enhanced chlorophyll degradation (Tavakkoli et al. 2010) observed in this genotype. These results also corroborate with the significantly enhanced gene expression of *ClC-b2* and *ClC-c2* in LGG 460. Similar response was observed in salt-sensitive common bean (*Phaseolus vulgaris*) (Seemann and Critchley 1985) and soyabean (Läuchli and Wieneke 1979) grown in NaCl solution, that accumulated high Cl^- as compared to their respective tolerant varieties. In general, halophytes accumulate high levels of chloride ions (Cl^-) as it requires less energy to accumulate than to exclude Cl^- and can serve as an osmoticum under salt stress (Bazihizina et al. 2019). Cl^- exclusion in MGG 295 could be attributed to the efficient osmotic balance already achieved through enhanced uptake of Na^+ and K^+ ions. Therefore, excluding Cl^- helps minimize ion toxicity. Notably, several salt-tolerant non-halophyte like barley and soybean genotypes exhibit a similar strategy of Cl^- exclusion under saline conditions to

alleviate symptoms associated with Cl^- toxicity (Tavakkoli et al. 2011; Chen et al. 2013).

Notably, the relatively tolerant genotypes MGG 295 and MGG 351 employ distinct salt tolerance mechanisms, with MGG 351 relying on Na^+ exclusion and xylem unloading of Na^+ ions (significantly increased expression of root *SOS1* and leaf *HKT1* and reduced expression of root *HKT1*), sequestration (increased expression of leaf *NHX1*), and high ionic discrimination (selective K^+ uptake, potentially due to increased expression on leaf *AKT1*). In contrast, MGG 295 exhibits low ionic discrimination (significantly reduced expression of *SOS1* and *SOS2*). and implements stringent Cl^- exclusion strategies (decreased expression of *ClC-b2* and *ClC-c2*). A similar trend was also observed in rice genotype Kamini that phenotypically demonstrated salt tolerance similar to another genotype FL478 but accumulated high Na^+ content and was much less selective in K^+ uptake as compared to FL478 (Chakraborty et al. 2020). Authors ascribed it to the higher tissue tolerance for maintaining plant integrity under high Na^+ load (Chakraborty et al. 2020). A comparable mechanism of enhanced tissue tolerance towards Na^+ accumulation could be anticipated for MGG 295. This trait is typically assessed by monitoring chlorophyll degradation with increasing Na^+ concentration over a period of time (Yeo and Flowers 1983). Although this study did not assess continuous monitoring of Na^+ and chlorophyll content over time, yet we see minimal chlorophyll degradation despite high Na^+ accumulation in MGG 295. Further, ion compartmentalisation is central to tissue tolerance, though the expression of *NHX1* in MGG 295 does not indicate enhanced vacuolar Na^+ compartmentalisation. In salt-acclimated tobacco cells, *NHX1* and *NHX2* gene expression remained unchanged despite significant Na^+ influx into vacuoles. Thus, *NHX1* expression level is not sufficient to rule out ion sequestration. Indirect evidence for high Na^+ sequestration comes from the high cellular K^+ content under salt stress. In tolerant non-halophytes like barley high retention of cytosolic K^+ is essential to support the increased vacuolar Na^+ sequestration under high Na^+ load. This is associated with reduced accumulation of organic osmolytes resulting in less energy cost of stress survival and better growth (Chen et al. 2007a). On the same lines, high K^+ retention and low proline accumulation in MGG 295 suggest increase in Na^+ sequestration. There are other routes of Na^+ sequestration like the vacuolar salt deposition by extensive vesicle trafficking of Na^+ between the plasma membrane and the vacuolar compartments (De la Garma et al. 2015), that need to be explored in MGG 295. The tissue tolerance in MGG 295 also seem to operate through minimising energy cost of stress adaptation. Na^+ exclusion and selective K^+ uptake from rhizosphere is an energy requiring process (Huang et al. 2019). It is a major factor that effects plant performance under saline conditions (Munns et al. 2020) leading to trade-off between productivity and stress tolerance. It seems plausible that the tissue tolerance in MGG 295 is probably fuelled by channelling this energy towards plant maintenance under salinity load, which would otherwise be utilised in exclusion or selective transport.

It is also interesting to note that the salinity stress adaptations for ion imbalance in the tolerant MGG 295 and sensitive LGG 460 are reminiscent of adaptations towards acute and chronic salt stress conditions. Acute salt stress refers to sudden and short-term exposure to high levels of salinity during which the plant quickly excludes Na^+ from the cytosol or sequesters it into vacuoles, triggers short-term protective mechanisms like the synthesis of osmolytes (e.g., proline), antioxidant enzyme activation etc. as has been observed in LGG 460. This helps avoid immediate cellular damage, but the plant may not sustain these mechanisms for extended periods. Chronic salt stress refers to prolonged exposure to saline conditions during which plants must adopt long-term strategies to maintain ion homeostasis. This involves more efficient use of Na^+ sequestration into vacuoles and prioritizing K^+ uptake to balance ion toxicity as observed in MGG 295. This seems that MGG 295 prepares for a long-term stress condition early-on so as to keep the biological process functioning. This feature is of particular agronomic importance for crop improvement as breeding programs prioritize genotypes that can sustain long-term ion regulation under prolonged salinity that occur more frequently in field conditions like poorly managed irrigated fields or coastal soils (Munns and tester 2008; Flowers and Colmer 2008; Zhu 2001).

Conclusion

The study provides a comprehensive summary of physiological and molecular traits that can serve as effective tools for screening and evaluating different genotypes for stress adaptation. This information is critical for breeding programs aimed at develop8ing salt-tolerant varieties. Furthermore, the study emphasizes the complexity of mechanisms involved in salinity tolerance in mungbean, highlighting the distinct strategies employed by two tolerant genotypes to cope with stress. Our preliminary results suggest that salt tolerance in MGG 351 potentially operates through Na^+ extrusion and sequestration, akin to LGG 460, resulting in reduced Na^+ transport to leaf tissues. Conversely, MGG 295 exhibits low ionic discrimination, non-selective K^+ retention and Cl^- exclusion to minimise ion toxicity. These preliminary findings underscore the diversity of salinity tolerance strategies even within non-halophytic species like mungbean. From a breeding perspective, the distinct salt adaptations observed in the three genotypes provides valuable insights into some important salt tolerance traits that can

be harnessed for crop improvement programs. However, the complexity of these mechanisms also suggests that a one-size-fits-all approach may not be effective. The ongoing debate in the field of breeding for salinity tolerance revolves around whether to prioritize ion exclusion or tissue tolerance. The study highlights the importance of both strategies, showing that different genotypes may rely on one or the other, or even a combination of both, to thrive under saline conditions. It thus warrants to comprehensively decipher relative effectiveness of both the approaches that will encourage the re-evaluation of established paradigms, paving way for the novel innovative approaches to design salinity tolerant crops. Moreover, many ion transporters do not function in isolation but as part of larger protein complexes. Research should focus on the identification and functional characterization of these transporter complexes and interacting partners in ion homeostasis that could reveal new ways to enhance transporter efficiency through molecular breeding or biotechnological approaches. Concomitantly, further research is needed to explore the ion partitioning and sequestration specifically in tissue and organelle specific manner. This could be achieved by performing elemental analysis, their distribution and concentration in various cell types, cytoplasm, vacuoles, and other organelles and establishing their relation to tissue tolerance by measuring photosynthetic performance and key anatomical and morphological features. Quantitative X-ray microanalysis and Laser ablation connected with ICP-MS or ICP-OES are some of the excellent methods for direct in vivo determination of elemental distribution in plants that can provide cell- and organ-specific concentrations of different elements across roots and leaves and possibly decipher the control points for salt ion transport between root and shoot (Iqbal et al. 2024; Hanć et al. 2016). The implications of developing salt-tolerant mungbean is profound for global agriculture, particularly in regions affected by soil salinity. By improving productivity in saline soils, such crops have the potential to increase food security, enhance the resilience of farming systems, and mitigate the effects of land degradation. As soil salinity becomes an increasing challenge due to climate change and unsustainable agricultural practices, the development of salt-tolerant crops will be critical for ensuring agricultural sustainability and resilience in the future.

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Data availability Data will be made available upon request.

Declarations

Competing interests Authors declare no competing interest.

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