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Research article

Nitrogen alleviates salinity toxicity in *Solanum lycopersicum* seedlings by regulating ROS homeostasis

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ABSTRACT

The present study was aimed to investigate adaptation in physiology and biochemistry of *Solanum lycopersicum* seedlings under NaCl ($NaCl_0$; 0.0 g NaCl kg^{-1} sand, $NaCl_1$; 0.3 g NaCl/kg sand and $NaCl_2$; 0.5 g NaCl/kg sand) stress, simultaneously supplemented with different (deprived; 0 mg/kg sand, LN; 105 mg/kg sand, MN; 210 mg/kg sand and HN; 270 mg/kg sand) levels of nitrogen (N). NaCl at both doses caused significant loss in growth, K^+ content, K^+/Na^+ ratio, total chlorophyll and photosynthetic oxygen evolution. Further, N supplementation influences growth of test seedlings, that attained maximum growth in HN followed by MN, LN and deprived N conditions. N at HN level significantly declined Na^+ accumulation in the cell and enhanced level of K^+. NaCl treatment enhanced level of oxidative stress biomarkers: superoxide radical ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), MDA equivalents contents and electrolyte leakage in leaf as well as root despite enhanced activity of SOD, POD, CAT and GST, and enzymes participating in the ascorbate-glutathione cycle (AsA-GSH cycle) *viz.* APX, DHAR and GR. At the same time, higher contents of total AsA (AsA + DHA) and total GSH (GSH + GSSG), and maintained ratios of AsA/DHA and GSH/GSSG in HN fed seedlings were observed. Overall, the results suggest that HN supplementation was able in alleviating NaCl induced toxicity in test seedlings which was mainly due to the up-regulation of the AsA–GSH cycle, K^+ and K^+/Na^+ ratio, which resulted into better growth performance of HN fed seedlings under NaCl stress while reverse was noticed for LN and deprive N conditions.

1. Introduction

The weathering processes of rocks, flooding condition as well as anthropogenic activities such as urbanization, industrialization and irrational agricultural practices discharge every year a huge amount of salt in the soil and water of urban and peri-urban agricultural areas (Nurmakhanova et al., 2014; Singh et al., 2015a). According to the FAO (2011), almost 34 million hectares of irrigated lands throughout the world have been suffered by salinity. In addition to this, 60–80 million hectares lands are still facing condition of water logging and concerned salinity problems. Moreover, evidences showed that among numerous abiotic stresses, salinity considered as a major hurdle for crop productivity worldwide (Munns, 2002; Munns and Tester, 2008; Nath et al., 2016; Chattergee et al., 2018). Salinity causes an imbalance of cellular ions resulting in ion toxicity and osmotic stress simultaneously inducing production of reactive oxygen species (ROS) such as superoxide radicals ($O_2^{\bullet-}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and successively formed the most toxic hydroxyl radicals($^{\bullet}OH$) through Fenton reaction in plants (Halliwell and Gutteridge, 1985; Parida and Das, 2005; Singh et al., 2015a). These oxygen derivatives mainly formed via the leakage of electrons from the electron transport chains and may interact with cellular components such as proteins, lipids and DNA and causing disturbance in their configuration that shifted cells towards death (Imlay and Linn, 1988; Pastori and Foyer, 2002; Apel and Hirt, 2004; Nounjan et al., 2012; Yildiz and Terz, 2013).

At the same time, to survive under harsh environment plants have an efficient sets of enzymatic i.e. superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione-*S*-transferase (GST) etc. and non-enzymatic i.e. non protein thiols (NP-SH), cysteine, proline etc. antioxidants that maintain the production of ROS under threshold limit

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Abbreviations: APX, ascorbate peroxidase; AsA, reduced ascorbate; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; EL, electrolyte leakage; GSH, reduced glutathione; GR, glutathione reductase; GST, glutathione-*S*-transferase,H_2O_2: hydrogen peroxide; HN, high nitrogen; K, potassium; LN, low nitrogen; MDA, malondialdehyde; MN, medium nitrogen; N, nitrogen; Na, sodium; NaCl, sodium chloride; NP-SH, non-protein thiol; POD, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SOR, superoxide radical

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so that they could perform only vital developmental activity rather than damage. Furthermore in the series of ROS detoxification mechanism, enzymes involved in the ascorbate-glutathione cycle (AsA-GSH cycle) i.e. ascorbate peroxidase (APX), glutathione reductase (GR), monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR), and their associated metabolites i.e. ascorbate (AsA) and glutathione (GSH) play decisive role in H_2O_2 breakdown particularly (Noctor and Foyer, 1998; Singh et al., 2015b). These enzymes scavenge ROS from surrounding environment of the cell. In this cycle, H_2O_2 is reduced into H_2O with the help of ascorbate peroxidase (APX) using ascorbate (AsA) as the specific electron donor (Borella et al., 2019). Moreover, AsA and GSH are associated with the cellular redox balance which may function as signals for the regulation of antioxidant mechanisms (Mittler, 2002; Chattergee et al., 2018).

In order to meet demand of food by increasing population, alternative strategies are required to ease adverse effects of salt on crops. Chemical application and agronomical crop management practices have been used to alleviate the salt toxicity with some success. Exogenous application of nitrogen (N) may be an alternative policy to enhance plants capability to face successfully the harsh situation of salt toxicity. Nitrogen is essential macronutrient which is needed for growth and development in plant. It is integrated into several nitrogen containing compounds, such as amino acids, proteins, nucleic acids, plant hormones, osmolytes, vitamins and chlorophylls, etc. (Siddiqui et al., 2012; Singh et al., 2016; Sudmalis et al., 2018). Studies showed that N application improves plant growth by stimulating growth supporting metabolic activities in *Egeria densa* (Correia et al., 2005). In addition to this, N also plays an important role in the regulation of the AsA-GSH cycle by accelerating the biosynthesis of GSH via supplying nitrogenous amino acids such as cysteine, glutamic acid, and glycine (Mullineaux and Rausch, 2005). Comprehensive studies regarding impacts of salinity on plants are available, which exclusively devoted to the action site (s) of salinity on physiological and/or biochemical responses, however, there is no information available concerning the effect of N application on modulation of the AsA-GSH cycle in the presence or in the absence of salinity. Thus aim of the present study was to investigate salt tolerance strategies in stressed plants supplemented with N by evaluating enzymatic and non-enzymatic antioxidant defense system including the AsA-GSH cycle. The study was also aimed to establish a possible relationship between N application and oxidative stress tolerance in plants exposed to saline conditions.

2. Material and methods

2.1. Plant material, growth conditions and nitrogen treatments

The healthy seeds of *Solanum lycopersicum* L., var. Lakshmi (tomato) were procured from Nunhems Pvt. Ltd. India. Seeds were surface sterilized with 2% (v/v) sodium hypochlorite solution for 15 min and washed thoroughly with distilled water and left into distilled water for 4 h soaking period. Further, seeds were wrapped in sterilized cotton cloth and left overnight for germination at 25 ± 1 °C. Next day, sprouted seeds were sown in sand already mixed with three doses ($NaCl_0$; 0.0 g NaCl kg^{-1}, $NaCl_1$; 0.3 g NaCl kg^{-1} and $NaCl_2$; 0.5 g NaCl kg^{-1} sand) of NaCl in plastic pots (5 cm in diameter and 10 cm in depth) containing 150 g acid washed sterilized sand wet by the Hoagland nutrient medium (Hoagland and Arnon, 1950). Seedlings were placed in a growth chamber (CDR model GRW-300 DGe, Athens) under photosynthetically active radiation (PAR) of 350 μmol photons $m^{-2} s^{-1}$ with 16:8 h (light: dark cycle)along with 65–70% relative humidity at 25 ± 1 °C. After appearance of primary leaves, seedlings were subjected to different levels of N treatment.

After emergence of primary leaf (15 days of growth), NaCl treated and untreated seedlings were irrigated with full strength Hoagland nutrient medium containing different concentrations [0, 105, 210 and 270 N mg/kg sand which correspond to deprived, low nitrogen (LN), medium nitrogen (MN that concentration presents in normal full strength Hoagland medium) and high nitrogen (HN), respectively] of N, obtained from $CaNO_3.4H_2O$ (Table 1). The experimental set up includes 12 combinations: deprived + $NaCl_0$, deprived + $NaCl_1$, deprived + $NaCl_2$, LN + $NaCl_0$, LN + $NaCl_1$, LN + $NaCl_2$, MN + $NaCl_0$, MN + $NaCl_1$, MN + $NaCl_2$, HN + $NaCl_0$, HN + $NaCl_1$, HN + $NaCl_2$. Since we had to investigate effect of NaCl as well as combined effect of NaCl, during different N supplementation condition and we had to establish parity in terms of dose to find out the maximum yield of *S. lycopersicum*, we selected the above mentioned doses of N. In this way, the 75 kg N ha^{-1}sand which corresponds to MN that was reported to environmentally realistic dose and also present in normal full strength Hoagland medium that's why MN served as control throughout the experiment. The test seedlings were irrigated regularly with different levels of N at every 3 day of interval, until 26 days old seedlings and at 30 day of growth seedlings were harvested for the analysis of various parameters.

Table 1

Composition of a modified Hoagland and Arnon (1950) nutrient solution for different level of N.

Compounds	Elements	Final Concentration of elements mg^{-1} kg sand			
		N-deprived	LN	MN	HN
Macronutrients					
$Ca(NO_3)2{\cdot}4H_2O$	N	0	105	210	270
KCl (in place of KNO_3)	K	235	235	235	235
$CaCl_2$ (for valancing remaining Ca)	Ca	200	200	200	200
$NH_4H_2PO_4$	P	31	31	31	31
$MgSO_4{\cdot}7H_2O$	S	64	64	64	64
	Mg	48	48	48	48
Micronutrients					
H_3BO_3	B	0.5	0.5	0.5	0.5
$MnSO_4{\cdot}H_2O$	Mn	0.5	0.5	0.5	0.5
$ZnSO4{\cdot}7H_2O$	Zn	0.05	0.05	0.05	0.05
$CuSO_4{\cdot}5H_2O$	Cu	0.02	0.02	0.02	0.02
H_2MoO_4 (85% MoO_3)	Mo	0.01	0.01	0.01	0.01
NaFeDTPA (10% Fe)	Fe	1.0–5.0	1.0–5.0	1.0–5.0	1.0–5.0

2.2. Estimation of growth and photosynthetic pigments

Growth was determined by measuring fresh and dry weight of shoot and root. Fresh weight was measured using digital electronic weighing balance (Contech-CA 223, India). Shoot and root length was measured using centimetre scale. For dry weight measurement, shoot and root were oven dried at 80 °C for 48 h than weighed by using electronic balance. Photosynthetic pigments were determined as per method given by Lichtenthaler (1987).

2.3. Estimation of Na^+ and K^+ contents

Elemental (Na and K) analysis was carried out according to the method of Allen et al. (1986) using atomic absorption spectrometer (iCE 3000 Series, model-3500 AAS, Thermo scientific, UK), fitted with specific lamp of particular metal using appropriate drift blank.

2.4. Estimation of gross photosynthesis rate

The gross photosynthesis rate was measured in terms of the sum of oxygen evolution (photosynthesis rate) and consumption (dark respiration rate) from 50 mg fresh leaves/leaf discs without mid veins in presence and absence of light, respectively using Clark type oxygen electrode (Digital Oxygen System, Model-10, Rank Brothers, UK) as

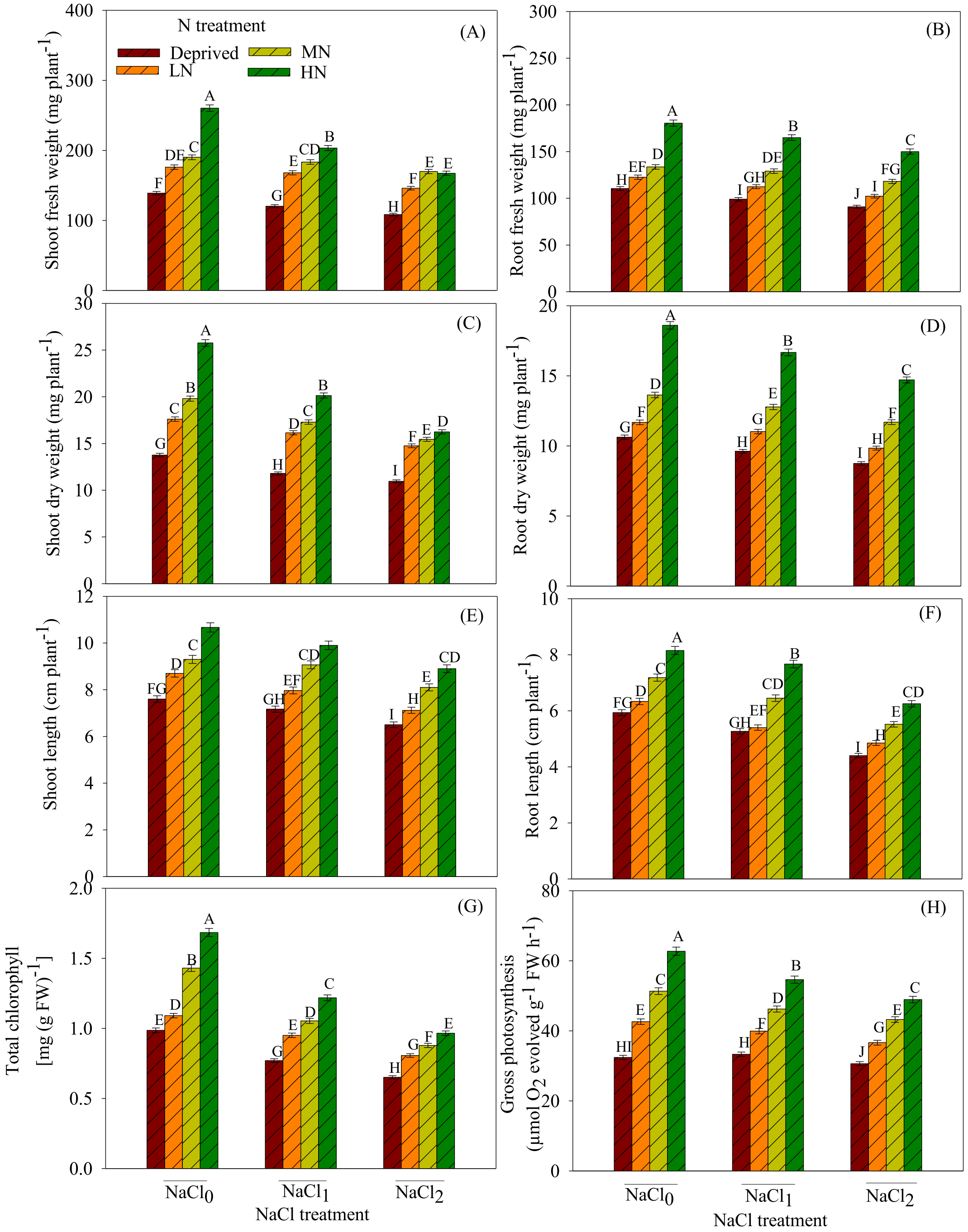

Fig. 1. Effect of different levels of N on shoot and root fresh and dry weight, shoot and root length, total chlorophyll (Chl *a* + Chl *b*) and photosynthetic oxygen evolution in *Solanum lycopersicum* seedlings under NaCl stress. Data are means ± standard error of three replicates (n = 3). Each bar followed by different letter (s) show significant differences at $P < 0.05$ among different treatments according to the Duncan's multiple range test.

described by Kurra-Hotta et al. (1987). The gross photosynthesis rate were expressed as µmol oxygen evolved g^{-1} FW h^{-1}.

2.5. Estimation of oxidative stress biomarkers in vitro and in vivo

In vitro and *in vivo* estimation of superoxide radicals (SOR; $O_2^{\bullet -}$) was carried out by Elstner and Heupel (1976) and Frahry and Schopfer (2001), respectively. *In vitro* and *in vivo* measurement of hydrogen peroxide (H_2O_2) was conducted according to the methods of Velikova et al. (2000) and Thordal-Christensen et al. (1997), respectively. *In vitro* and *in vivo* determination of lipid peroxidation was done according to the methods of Hodges et al. (1999) and Pompella et al. (1981), respectively. *In vitro* and *in vivo* estimation of membrane damage was carried out by the methods of Gong et al. (1998) and Yamamoto et al. (2001), respectively.

2.6. Estimation of enzymatic antioxidants

Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured according to the method of Giannopolitis and Reis (1977). One unit (U) of SOD activity is defined as the amount of enzyme required to cause 50% inhibition in reduction of NBT.

Peroxidase (POD; EC 1.11.1.7) activity in leaves and roots of each set of seedlings was determined according to the method of Zhang

(1992). Enzyme activity was calculated by using an extinction coefficient 25.5 mM^{-1} cm^{-1}. One unit (U) of POD activity is the amount of enzyme oxidizing 1 μmol guaiacol mg^{-1} protein min^{-1}.

Catalase (CAT; EC 1.11.3.6) activity was assayed in terms of decrease in absorbance due to dissociation of H_2O_2, which was recorded at 240 nm and quantified by using an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ (Aebi, 1984). One unit (U) of enzyme activity is defined as 1 μmol H_2O_2 dissociated mg^{-1} protein min^{-1}.

Glutathione-*S*-transferase (GST, EC 2.5.1.18) activity was measured following the method of Habig et al. (1974). Enzyme activity was calculated by using an extinction coefficient 9.6 mM^{-1} cm^{-1}. One unit (U) of enzyme activity is defined as 1 μmol of CDNB-conjugates formed mg^{-1} protein min^{-1}.

2.7. Estimations of components of AsA-GSH cycle

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was determined according to the method of Nakano and Asada (1981). The decrease in absorbance due to oxidation of ascorbate was recorded at 290 nm and the enzyme activity was calculated by using an extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. One unit (U) of enzyme activity is defined as 1 μmol ascorbate oxidized mg^{-1} protein min^{-1}.

Glutathione reductase (GR; EC 1.6.4.2) activity was determined by the method of Schaedle and Bassham (1977). The decrease in absorbance was recorded at 340 mM by UV – VIS spectrophotometer (Evolution 201, Thermo scientific, UK) for 3 min at 25 °C. The GR activity was calculated using the extinction coefficient $\varepsilon = 6.2$ $mM^{-1}cm^{-1}$. One unit of enzyme activity is defined as 1 μmol NADPH oxidized mg^{-1} protein min^{-1}.

Dehydroascorbate reductase (DHAR; EC 2.5.1.18) activity was assayed by monitoring the increase in absorbance at 265 nm as a result of reduction of dehydroascorbate (DHA) into reduced ascorbate (AsA) using an extinction coefficient of 7.0 mM^{-1} cm^{-1} (Nakano and Asada, 1981). One unit of enzyme activity is defined as 1 μmol DHA reduced mg^{-1} protein min^{-1}.

Total ascorbate (AsA + DHA), ascorbate (AsA) and dehydroascorbate (DHA) contents were determined by the method of Gossett et al. (1994). For each sample, DHA was obtained by deducting the values of AsA from AsA + DHA. Ascorbate content was calculated by using standard curve prepared with L-ascorbic acid. Total glutathione, GSSG and GSH contents were estimated according to method of Brehe and Burch (1976). The amount of GSH was determined by subtracting GSSG from GSH + GSSG using a standard curve prepared with GSH.

2.8. Statistical analysis

Results were statistically analyzed by analysis of variance (ANOVA). Duncan's multiple range test was applied for mean separation for significant differences among treatments at $P < 0.05$ significance level. The results presented are the means ± standard error of three replicates ($n = 3$).

3. Results

3.1. Impact of N on growth parameters under NaCl stress

Growth was measured in terms of fresh and dry weight and lengths of shoots and root of *Solanum lycopersicum* seedlings exposed to NaCl ($NaCl_0$; 0.0 g NaCl kg^{-1}, $NaCl_1$; 0.3 g NaCl kg^{-1} and $NaCl_2$; 0.5 g NaCl kg^{-1} sand) under varying concentrations (Deprived; 0 mg/kg, LN; 105 mg/kg, MN; 210 mg/kg and HN; 270 mg/kg sand) of N (Fig. 1). As compared to the control (MN without NaCl), maximum growth was observed in HN level of N treated seedlings, while it was the least observed in deprived level of N. Maximum decline in shoot and root fresh and dry weight was observed under combined treatment of $NaCl_2$ and deprived level of N. In contrast to this, HN level of N alone stimulated shoots and root fresh weight by 37 and 35%, shoots and root dry weight by 40 and 36%, and root and shoots length by 15 and 13%, respectively. Furthermore, significant attenuation of NaCl toxicity was observed in all growth parameters under HN level of N as compared to the control (Fig. 1).

3.2. Impact of N on photosynthetic pigments under NaCl stress

The results related to photosynthetic pigments as total chlorophyll (Chl *a* + *b*) are shown in Fig. 1. As compared to the control (MN without NaCl), MN treated seedlings under $NaCl_1$ and $NaCl_2$ doses showed considerable reduction in total Chl. NaCl treatment more severely affected contents of total Chl when seedlings were supplemented with either LN or without exogenous N (deprived) application. The effect of $NaCl_1$ and $NaCl_2$ stress on total Chl content was minimized considerably when seedlings were supplemented with HN (Fig. 1).

3.3. Impact of N on Na^+ and K^+ contents and K^+/Na^+ ratio under NaCl stress

The results pertaining to Na^+ and K^+ contents and K^+/Na^+ ratio are presented in Fig. 2. As compared to the control (MN without NaCl), MN supplemented seedlings with $NaCl_1$ and $NaCl_2$, showed enhancement in Na^+ accumulation whereas, K^+ concentration as well as K^+/Na^+ ratio exhibited a decreasing trend, as K^+ content was reduced by 19 and 31% in shoot and 10 and 21% in root, and K^+/Na^+ ratio was decreased by 62 and 75% in shoot and 63 and 76% in root, respectively (Fig. 2). In addition to this, the progressive rise in Na^+ content was noticed in *S. lycopersicum* under LN + $NaCl_1$ and LN + $NaCl_2$ combinations while reverse trend was observed in the case of K^+ content and K^+/Na^+ ratio. On contrary to this, when the seedlings were supplemented with HN, the significant decrease was observed in Na^+ contents, while K^+ contents and K^+/Na^+ ratio both exhibited an enhancement trend, in shoot and root under NaCl stress as compared to the respective controls (Fig. 2).

3.4. Impact of N on gross photosynthesis under NaCl stress

The data pertaining to gross photosynthesis is shown in Fig. 1. The results demonstrate that exposure of tomato seedlings to NaCl caused significant loss in gross photosynthesis, which was further influenced by different levels of N. The maximum photosynthetic oxygen yield was noticed under higher concentration (HN) of nitrogen.

3.5. Impact of N on oxidative stress biomarkers under NaCl stress

The data pertaining to the $O_2^{\bullet -}$ (SOR; superoxide radical), hydrogen peroxide (H_2O_2), MDA and electrolyte leakage have been depicted in Fig. 3. As compared to the control (MN without NaCl), under MN + $NaCl_1$ and MN + $NaCl_2$ combinations *in vitro* measured SOR content increased considerably (Fig. 3A and B). Though under HN supplementation with NaCl stress a considerable rise in SOR and H_2O_2 contents was noticed however, reduction in growth was the least indicating that their levels were under control (Fig. 3A–D). Similar trend was noticed for MDA and electrolyte leakage under these treatments (Fig. 3E–H).

Similar to the results of *in vitro* analyses of oxidative stress biomarkers, *in vivo* analyses oxidative stress markers such as $O_2^{\bullet -}$, H_2O_2, MDA and electrolyte leakage showed more or less similar pattern (Figs. 4 and 5).

3.6. Impact of N on the status of enzymatic antioxidants under NaCl stress

The results reveal that N starvation in root as well as in leaf system appears itself as a limiting factor as all the studied enzymes activity

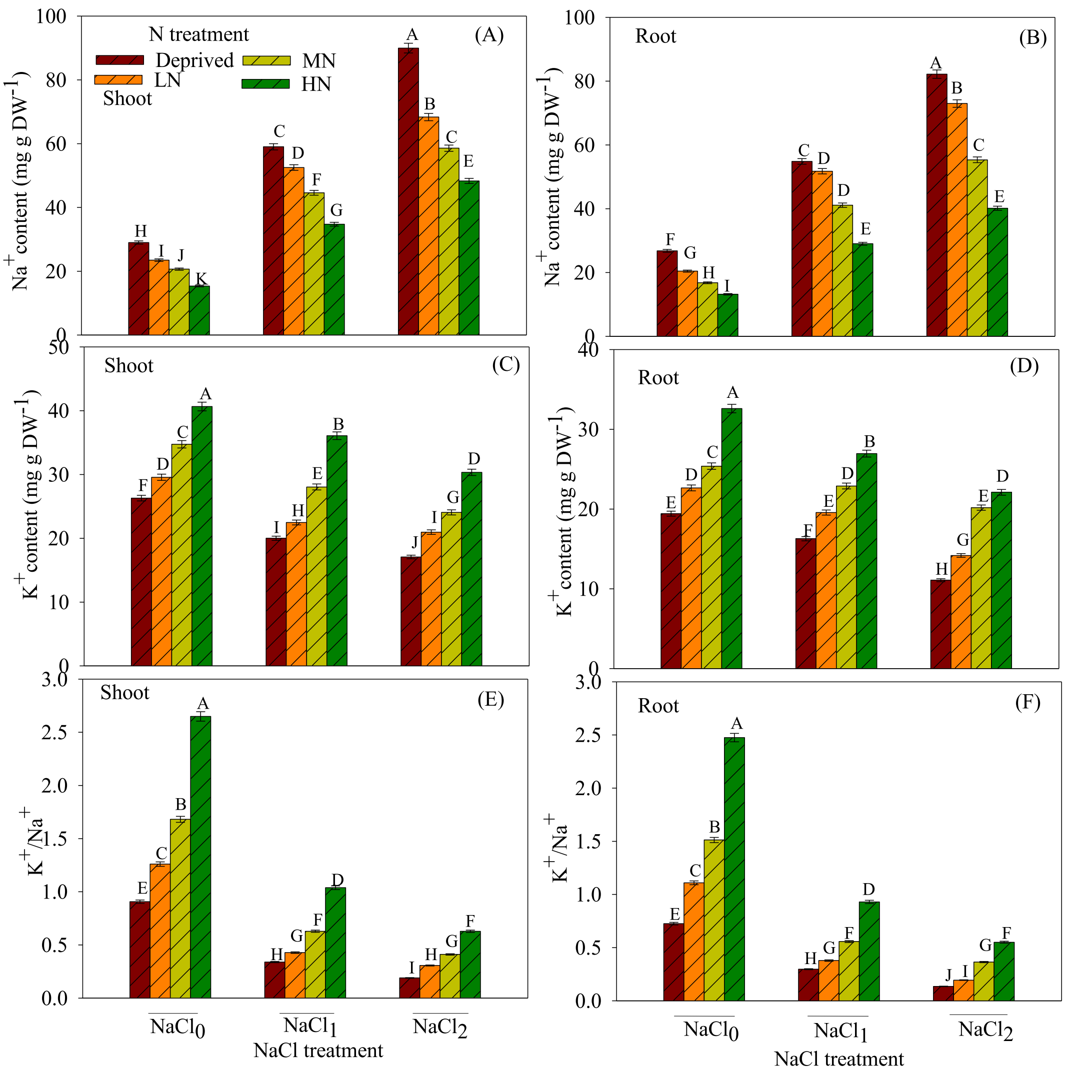

Fig. 2. Effect of different levels of N on sodium (Na^+) and potassium (K^+) contents and potassium/sodium (K^+/Na^+) ratio in *Solanum lycopersicum* seedlings under NaCl stress. Data are means ± standard error of three replicates (n = 3). Each bar followed by different letter(s) show significant differences at $P < 0.05$ among different treatments according to the Duncan's multiple range test.

showed decreasing trend being the lowest in deprived level of N under single treatment or in combination with NaCl (Fig. 6). In contrast to this, HN level of N significantly ($P < 0.05$) enhanced activity of SOD, POD, CAT and GST enzymes with the increasing concentration of NaCl as compared to the control. Further, the results also indicated that all the enzymes worked efficiently in leaf than root (Fig. 6).

3.7. Impact of N on components of the AsA-GSH cycle under NaCl stress

As compared to the control (MN without NaCl), MN supplemented seedlings under $NaCl_1$ and $NaCl_2$ stress caused substantial enhancement in activities of APX by 17 and 28%, GR by 10 and 14% and DHAR by 14 and 28% in *S. lycopersicum*, respectively. Conversely, seedlings supplemented with LN exhibited almost declining trend, and further activities of these enzymes showed maximum decline in deprived N. However, HN level of N supplemented seedlings under $NaCl_1$ and $NaCl_2$ dose showed accelerated activities of APX by 26 and 40%, GR by 18 and 27% and DHAR by 37 and 63% respectively as compared to the control (MN without NaCl) (Table 2).

The results pertaining to AsA and GSH, and their reduced and oxidized forms as well as their ratios are shown in Table 2. As compared to the control (MN without NaCl), total ascorbate (AsA + DHA) and reduced ascorbate (AsA) contents and ratio of AsA/DHA were significantly ($P < 0.05$) decreased in NaCl concentration dependent manner while DHA content showed increasing trend (Table 2). Further, upon LN as well as deprived level of N supplementation singly and in combination of $NaCl_1$ and $NaCl_2$ doses significantly declined AsA and GSH (except AsA/DHA). In contrast to this, AsA and GSH showed the highest values under HN supplementation without NaCl, however, with both doses of NaCl values of these parameters declined significantly but were still greater than LN and deprived N.

4. Discussion

In the present study, application of varying levels of N (deprived; 0 mg/kg sand, LN; 105 mg/kg sand, MN; 210 mg/kg sand and HN; 270 mg/kg sand) caused differential impact on growth, photosynthetic pigments and photosynthetic oxygen evolution, ion (Na^+ and K^+) accumulation, oxidative stress biomarkers and antioxidants enzymes/contents of *Solanum lycopersicum* seedlings grown under NaCl ($NaCl_0$; 0.0 g NaCl kg^{-1}, $NaCl_1$; 0.3 g NaCl kg^{-1} and $NaCl_2$; 0.5 g NaCl kg^{-1} sand) stress. The present study showed that growth, photosynthetic pigments and oxygen evolution significantly decreased after $NaCl_1$ and $NaCl_2$ treatments which could be directly correlated with proportional increase of Na^+ content in seedlings leading to enhanced Na^+ content in the cell (Siddiqui et al., 2012; Singh et al., 2016) (Fig. 2), decreased

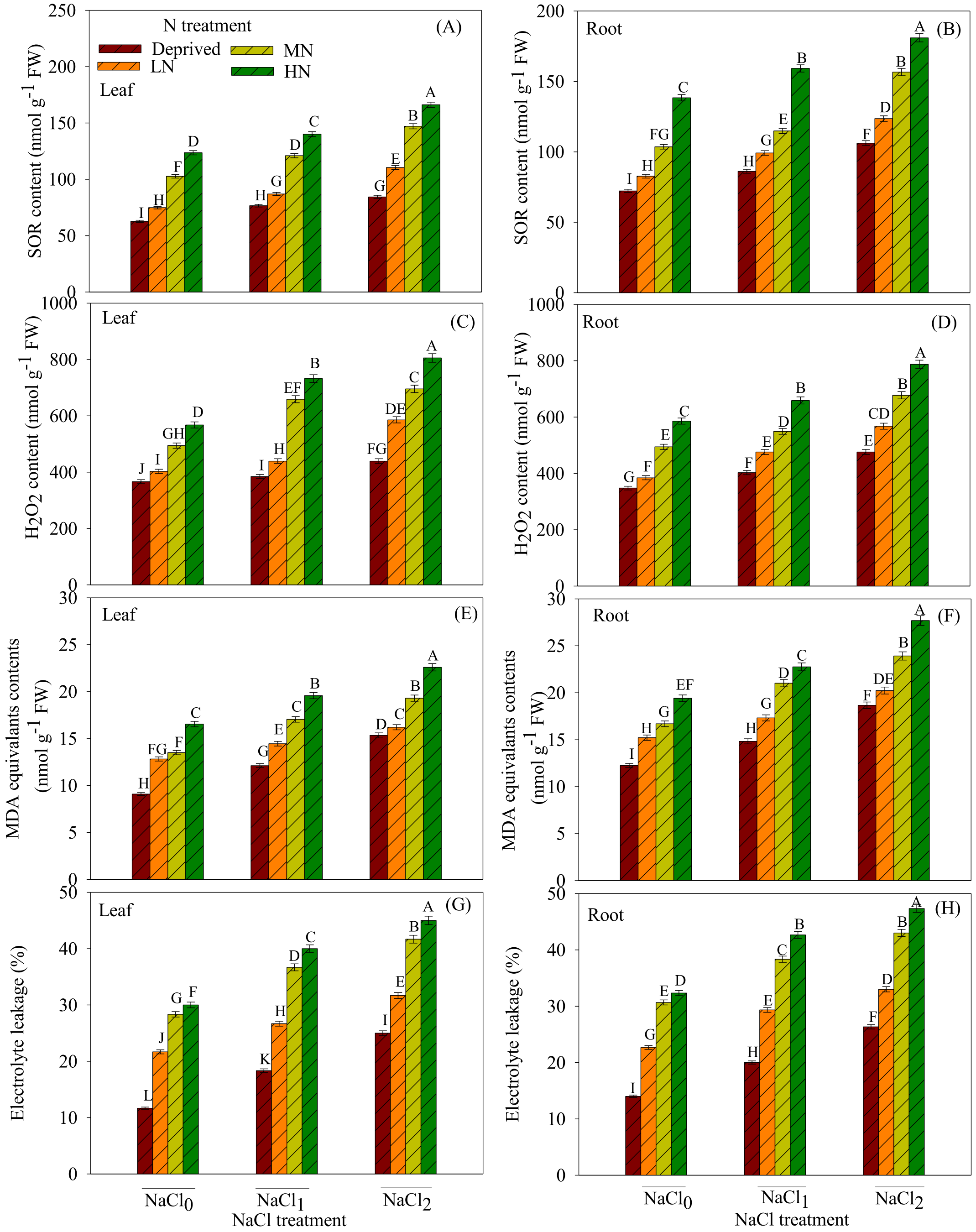

Fig. 3. Effect of different levels of N on superoxide radical (SOR) and hydrogen peroxide (H_2O_2), malondialdehyde (MDA) equivalents contents and electrolyte leakage (EL) levels in *Solanum lycopersicum* seedlings under NaCl stress. Data are means ± standard error of three replicates (n = 3). Each bar followed by different letter(s) show significant differences at $P < 0.05$ among different treatments according to the Duncan's multiple range test.

K^+ content as well as ratio of K^+/Na^+ in root and shoot (Fig. 2), along with overproduction of ROS (Figs. 3–5). Similarly, seedlings were supplemented with either LN or deprived level of N under NaCl stress significantly ($P < 0.05$) declined growth attributes by inhibiting rate of photosynthetic oxygen evolution (Fig. 1) due to no and/or low availability of N in test seedlings, as adequate amount of N is required in plant biological systems for numerous vital metabolic activities (Gong et al., 2013). In contrast to this, HN supplementation stimulated growth and also alleviated NaCl induced toxicity by accelerating K^+ and ratio of K^+/Na^+, and the ascorbate-glutathione cycle (AsA-GSH cycle) (Fig. 2). Evidences showed that N supplementation in preferable amount significantly enhanced growth by stimulating photosynthetic pigments, photosynthetic activity, amino acid synthesis, osmolyte production, nitrogen metabolism/nutrient cycle and others life supporting activities occurring inside the cell (Siddiqui et al., 2012; Ford et al., 2016; Singh et al., 2016; Sudmalis et al., 2018). Our results are in consonance with the finding of Akram and Ashraf (2009) who had also reported enhanced growth of sunflower after N supplementation. High level of N triggers the synthesis of some osmolytes under salt stress condition. These osmoticum are ammonium containing compounds (glycine betaine, b-alanine betaine, choline-O-sulfate, etc.), sugars and sugar alcohols (trehalose, fructan, mannitol, sorbitol, etc.) and amino acids (proline, etc.) play significant role in osmotic adjustment under salinity stress (Siddiqui et al., 2012; Yang et al., 2013; Singh et al., 2015a). Under salt stress condition, these compatible solutes maintain turgor pressure inside the cell by compartmentalizing high

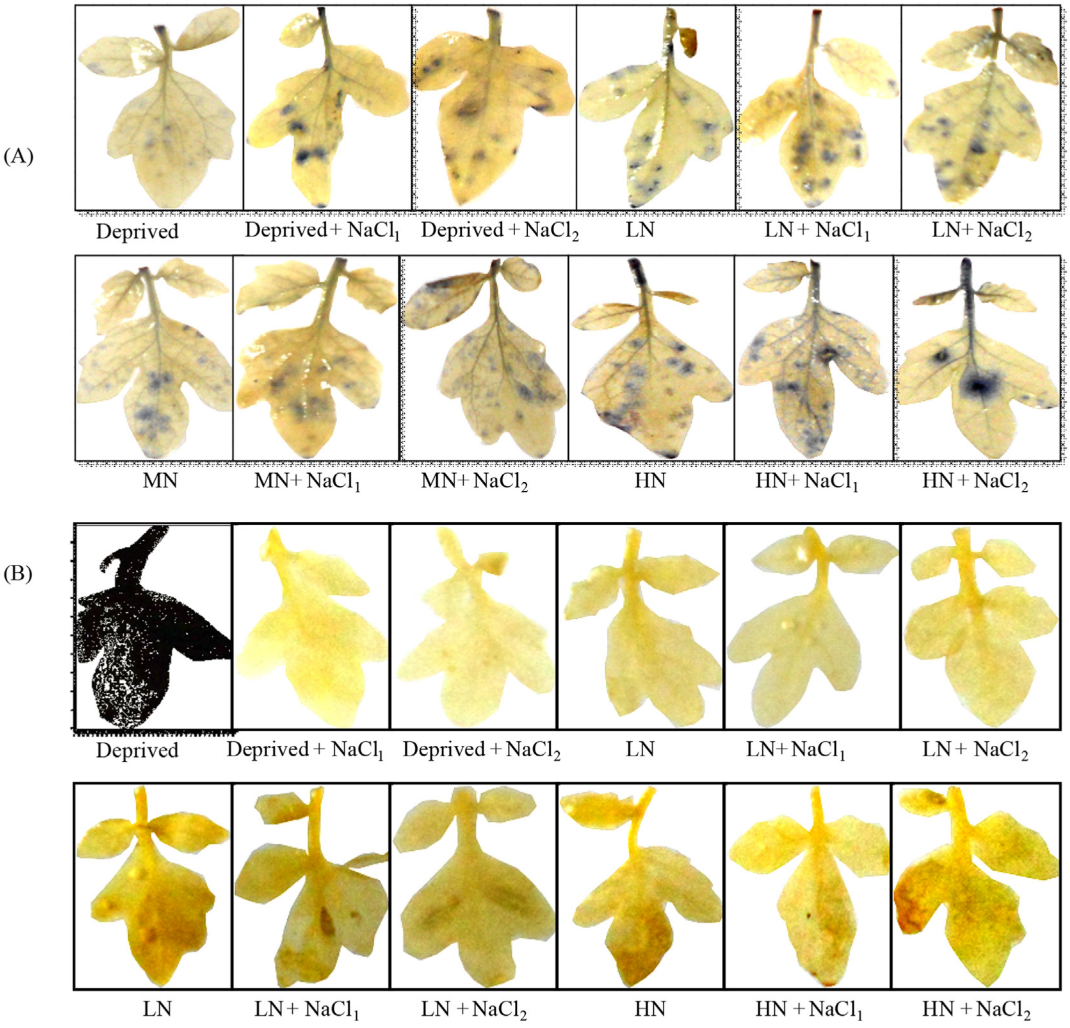

Fig. 4. *In vivo* visualization of SOR content (A) in the leaves of NaCl stressed and unstressed seedlings of *Solanum lycopersicum* supplemented with different levels of nitrogen. *Solanum lycopersicum* leaves stained with NBT and blue areas show SOR content. *In vivo* visualization of H_2O_2 content (B) in the leaves of NaCl stressed and unstressed seedlings of *Solanum lycopersicum* supplemented with different levels of nitrogen. *Solanum lycopersicum* leaves stained with DAB and brown areas show H_2O_2 content. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

concentrations of Na^+ and Cl^- in the vacuole (Munns, 2002; Munns and Tester, 2008; Polash et al., 2018). Similarly, Agami et al. (2018) reported that exogenous nitrogen supply mitigated adverse effect of stress in wheat plant by maintaining higher relative water content, water use efficiency, antioxidant system, osmoprotectants including proline while lowering relative membrane permeability. Besides the role of osmotic adjustment, proline also plays other roles like scavenging of radicals ($^{\bullet}OH$), stabilizing sub-cellular structures such as membranes and proteins, and buffering cellular redox potential under salt stress condition (Yang et al., 2013; Singh et al., 2015a; Khanna-Chopra et al., 2019).

Besides negative consequences of NaCl on growth supporting routes, stressors may also induce the risk of oxidative damage by stimulating production of ROS in plants cell in surplus amount. In the present study too, overproduction of ROS might have occurred due to strong inhibition of photosynthetic activities (Fig. 1) which cause damage to lipids and membranes in seedlings (Figs. 3–5). The stimulation of ROS was coincided with an increase in the lipid peroxidation as evident by enhanced MDA equivalents contents and thus accelerated electrolyte leakage (Fig. 3). ROS can rapidly interact with vital macromolecules (lipids and proteins) of cells as well as several other key metabolic enzymes and produce damaging effects and thus, resulted into a declined growth and deteriorating photosynthetic performance in *S. lycopersicum* seedlings. Furthermore, N supplementation also influences ($P < 0.05$) production/accumulation of ROS and their consequent damage to lipids and membrane, and electrolyte leakage in *S. lycopersicum* seedlings (Figs. 3–5). As compared to the control (MN without NaCl), under salt stress and HN treated seedlings showed more biomass accumulation as well as photosynthetic activity in spite of higher amount of ROS, indicating that under this treatment level of ROS was under control of antioxidants (Figs. 1, 3–6; Table 2).

Under adverse conditions, plants cope up with oxidative damage by up-regulating strong enzymatic and non-enzymatic antioxidants (Parida and Das, 2005; Yildiz and Terz, 2013; Singh et al., 2015a; Nath et al., 2016). In the present study, MN fed seedlings under $NaCl_1$ and $NaCl_2$ stress showed substantial enhancement in activities of SOD, POD, CAT and GST as compared to the control (Fig. 6). However, this increase in activities of enzymes was not sufficient to detoxify the ROS, hence NaCl induced damaging effect was noticed. Contrary to this, in case of HN supplemented seedlings though higher contents of ROS was observed but it had appeared that they do not damage seedlings due to simultaneous removal of ROS as evident by efficient activities of SOD, POD, CAT and GST enzymes. N-mediated enhancement in SOD activity accelerated scavenging of superoxide radicals ($O_2^{\bullet-}$) from photosynthetic and respiratory pathways. Likewise GST catalyzes conjugation of different electrophilic substrates with reduced glutathione and plays

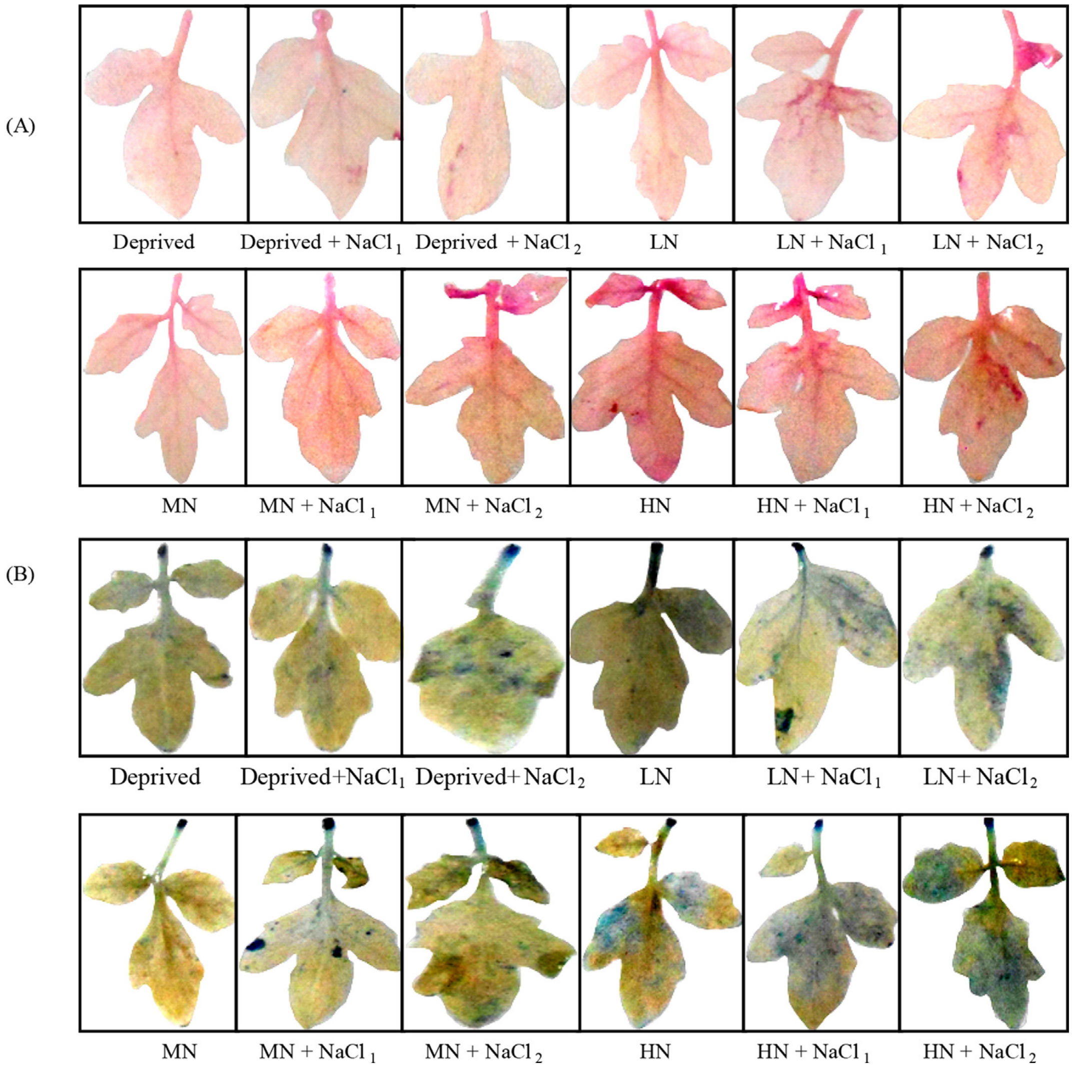

Fig. 5. *In vivo* visualization of MDA equivalents content (A) in the leaves of NaCl stressed and unstressed seedlings of *Solanum lycopersicum* supplemented with different levels of nitrogen. *Solanum lycopersicum* leaves stained with Schiff's reagent and pink areas show MDA equivalents contents. *In vivo* visualization of membrane leakage (B) in the leaves of NaCl stressed and unstressed seedlings of *Solanum lycopersicum* supplemented with different levels of nitrogen. *Solanum lycopersicum* leaves stained with Evan's blue and blue areas show membrane leakage. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

decisive role in detoxification of xenobiotics (Yadav et al., 2014). Thus, N-mediated enhanced level of SOD, POD, CAT and GST in *S. lycopersicum* seedlings might have acted as a protective mechanism under NaCl stress. The substantial increases in these enzymatic antioxidants are in similar with earlier findings of Misra and Gupta (2006) and Yañez-Mansilla et al. (2014).

Beside various enzymatic antioxidants, the AsA-GSH cycle enzymes i.e. APX, GR, MDHAR and DHAR as well as metabolites such as ascorbate and glutathione participate in ROS scavenging mechanism particularly for H_2O_2. In the present study, N mediated enhancement in APX activity under NaCl stress indicates that APX plays an important role in mitigating H_2O_2 induced oxidative damage (Table 2). The first step of the AsA-GSH cycle is H_2O_2 scavenging with the help of APX (Noctor and Foyer, 1998). Davletova et al. (2005) stated that absence of cytosolic APX caused disintegration of chloroplastic H_2O_2-scavenging system. Similarly, GR is another important enzyme of the AsA-GSH cycle that catalyzes NADPH-dependent reduction of GSSG into GSH. Chattergee et al. (2018) reported that GR enzyme plays important role in balancing GSH/GSSG ratio and this ratio acts as a redox buffer by participating in signalling as well as ROS scavenging mechanism under NaCl stress condition. GR activity showed similar response like that of APX against various doses of NaCl and different levels of N and their combinations (Table 2). However, decrease in GR activity under NaCl stress might be due to increased oxidative damage (Figs. 3–5) as well as may also depend on the status of NADPH because it is a product of non-cyclic photophosphorylation i.e. from photosynthesis. On the other hand, increase in GR activity under HN supplementation may enrich ROS scavenging process and thus avoided accumulation of ROS and minimized the risk of oxidative stress. Likewise, enhanced GR activity under HN treated *S. lycopersicum* seedlings suggests that high GSH content was maintained in tissues (Table 2), and made it available for successive DHAR enzyme activity. Similar to GR activity, as compared to the control, N mediated DHAR activity was also increased in *S. lycopersicum* seedlings on exposure to HN with or without NaCl treatment (Table 2). Enhanced DHAR enzyme activity might be explained as; DHAR uses GSH as electron donor, and facilitates AsA as a reductant to APX enzyme for scavenging of H_2O_2 and also balanced high AsA/DHA couple that plays important role in minimizing the risk of oxidative injury caused by NaCl (Noctor and Foyer, 1998). Borella et al. (2019) reported that nitrogen source enhances the enzymatic and non-enzymatic system of soybean plant. Noticeably, different levels of N together with both doses of NaCl increased activities of APX, DHAR and GR of

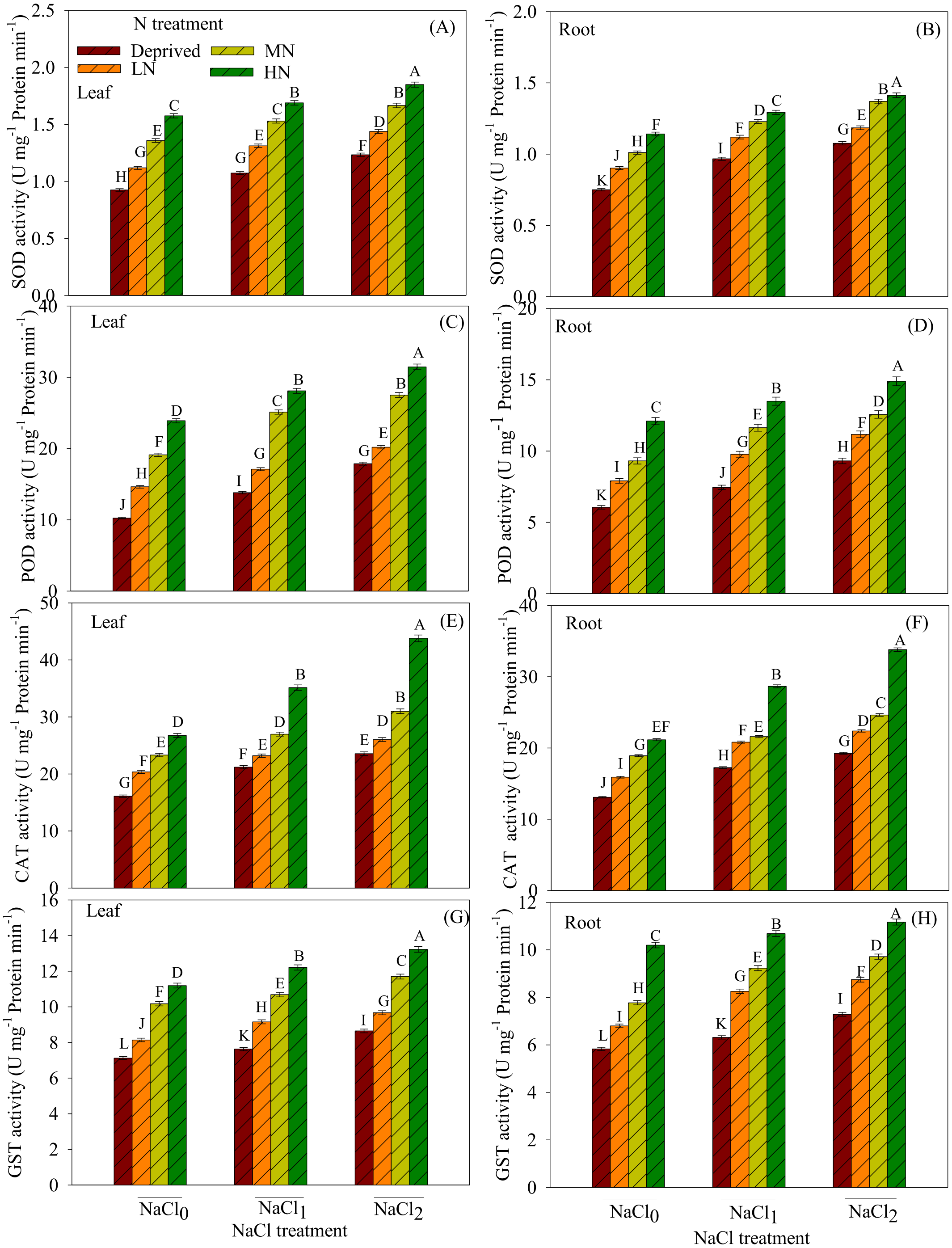

Fig. 6. Effect of different levels of N on activity of superoxide dismutase (SOD), peroxidase (POD) catalase (CAT) and glutathione-*S*-transferase (GST) in *Solanum lycopersicum* seedlings under NaCl stress. Data are means ± standard error of three replicates (n = 3). Each bar followed by different letter(s) show significant differences at $P < 0.05$ among different treatments according to the Duncan's multiple range test.

AsA-GSH cycle and its associated metabolites and being greater in HN (Table 2) which indicate their important role in detoxification of ROS under NaCl toxicity as evident from better growth, and this maybe one of the reasons for HN-mediated alleviation of NaCl toxicity (Fig. 1).

5. Conclusion

In conclusion, the present study showed that exogenous supplementation of N (deprived to HN) definitely modulated growth of *Solanum lycopersicum* seedlings under NaCl stress. Our study indicated that NaCl and N at LN and deprived N decreased growth of seedlings either in their single treatment or in their combinations. These declines in growth may be attributed to decrease in K^+ and K^+/Na^+, photosynthetic performance and AsA-GSH cycle, and higher accumulation of Na^+ and oxidative biomarkers. Conversely, HN supplementation with NaCl was found to alleviate NaCl toxicity due to improved performance of enzymes participating in the AsA-GSH cycle and concerned metabolites (ascorbate and glutathione) Furthermore, this study establishes the fact that N supplementation may play crucial role in managing NaCl stress, based on its exogenous concentrations and plant species under specific developmental and environmental conditions.

Table 2

Effect of different levels of N on activity of ascorbate peroxidase (APX), glutathione reductase (GR) and dehydroascorbate reductase (DHAR) and contents of ascorbate (AsA), dehydroascorbate (DHA), glutathione (reduced) and oxidized glutathione (GSSG) in *Solanum lycopersicum* seedlings under NaCl stress.

Enzymatic antioxidants (U mg^{-1} protein min^{-1})					Non enzymatic antioxidants (μmol g^{-1} DW)							
N treatment	NaCl treatment	APX	GR	DHAR	AsA + DHA	AsA	DHA	AsA/DHA	GSH + GSSG	GSH	GSSG	GSH/GSSG
Deprived	$NaCl_0$	96.61 ± 1.22^{g}	25.66 ± 0.31^{i}	0.15 ± 0.002^{i}	71.7 ± 1.3^{g}	44.9 ± 0.8^{f}	26.8 ± 0.5^{h}	1.67 ± 0.03^{ab}	2.16 ± 0.34^{d}	1.61 ± 0.25^{d}	0.54 ± 0.009^{f}	2.96 ± 0.04^{a}
	$NaCl_1$	116.66 ± 1.48^{e}	26.51 ± 0.31^{i}	0.19 ± 0.002^{g}	68.8 ± 1.3^{gh}	35.6 ± 0.7^{g}	33.1 ± 0.6^{g}	1.08 ± 0.02^{e}	1.37 ± 0.22^{gh}	0.93 ± 0.14^{gh}	0.43 ± 0.007^{h}	2.15 ± 0.03^{e}
	$NaCl_2$	129.42 ± 1.59^{d}	29.03 ± 0.35^{gh}	0.22 ± 0.002^{e}	64.4 ± 1.2^{h}	23.9 ± 0.4^{h}	40.4 ± 0.8^{e}	0.59 ± 0.01^{h}	1.02 ± 0.16^{i}	0.69 ± 0.10^{i}	0.32 ± 0.005^{j}	2.13 ± 0.03^{e}
LN	$NaCl_0$	109.37 ± 1.38^{f}	28.19 ± 0.34^{h}	0.16 ± 0.002^{h}	86.9 ± 1.6^{e}	54.1 ± 1.0^{d}	32.7 ± 0.6^{g}	1.65 ± 0.03^{b}	2.31 ± 0.37^{c}	1.72 ± 0.26^{c}	0.58 ± 0.009^{de}	2.93 ± 0.04^{a}
	$NaCl_1$	125.77 ± 1.59^{d}	29.45 ± 0.35^{g}	0.20 ± 0.003^{fg}	84.6 ± 1.6^{ef}	47.9 ± 0.9^{e}	36.8 ± 0.6^{f}	1.30 ± 0.02^{d}	1.94 ± 0.31^{e}	1.44 ± 0.22^{e}	0.49 ± 0.008^{g}	2.90 ± 0.04^{a}
	$NaCl_2$	142.18 ± 1.80^{c}	31.98 ± 0.38^{e}	0.22 ± 0.003^{de}	81.3 ± 1.5^{f}	37.2 ± 0.7^{g}	44.1 ± 0.8^{d}	0.84 ± 0.01^{g}	1.28 ± 0.20^{h}	0.88 ± 0.13^{h}	0.40 ± 0.006^{i}	2.17 ± 0.03^{e}
MN	$NaCl_0$	118.48 ± 1.50^{e}	30.71 ± 0.37^{f}	0.21 ± 0.002^{f}	105.6 ± 1.9^{c}	66.9 ± 1.2^{b}	38.6 ± 0.7^{ef}	1.73 ± 0.03^{a}	2.49 ± 0.40^{b}	1.78 ± 0.27^{b}	0.69 ± 0.011^{c}	2.56 ± 0.04^{b}
	$NaCl_1$	138.53 ± 1.75^{c}	33.66 ± 0.40^{d}	0.23 ± 0.003^{d}	103.0 ± 1.9^{cd}	62.6 ± 1.2^{c}	40.4 ± 0.7^{e}	1.55 ± 0.03^{c}	2.09 ± 0.33^{d}	1.48 ± 0.23^{e}	0.61 ± 0.010^{d}	2.43 ± 0.04^{c}
	$NaCl_2$	151.29 ± 1.92^{b}	34.92 ± 0.42^{c}	0.26 ± 0.003^{c}	98.6 ± 1.8^{d}	48.2 ± 0.9^{e}	50.4 ± 0.9^{b}	0.96 ± 0.02^{f}	1.44 ± 0.23^{g}	0.96 ± 0.14^{g}	0.48 ± 0.008^{g}	2.00 ± 0.03^{f}
HN	$NaCl_0$	125.77 ± 1.59^{d}	33.24 ± 0.40^{d}	0.23 ± 0.003^{d}	130.3 ± 2.4^{a}	82.4 ± 1.5^{a}	47.8 ± 0.9^{c}	1.72 ± 0.03^{ab}	2.83 ± 0.45^{a}	1.98 ± 0.30^{a}	0.85 ± 0.014^{a}	2.33 ± 0.03^{cd}
	$NaCl_1$	149.47 ± 1.89^{b}	36.18 ± 0.43^{b}	0.28 ± 0.004^{b}	117.4 ± 2.2^{b}	65.5 ± 1.2^{b}	51.8 ± 0.9^{b}	1.26 ± 0.02^{d}	2.40 ± 0.38^{bc}	1.65 ± 0.25^{d}	0.74 ± 0.012^{b}	2.24 ± 0.03^{de}
	$NaCl_2$	165.88 ± 2.10^{a}	39.13 ± 0.66^{a}	0.33 ± 0.004^{a}	113.7 ± 2.1^{b}	54.8 ± 1.0^{d}	58.9 ± 1.1^{a}	0.93 ± 0.02^{f}	1.61 ± 0.26^{f}	1.04 ± 0.16^{f}	0.56 ± 0.009^{ef}	1.85 ± 0.03^{g}

Data are means ± standard error of three independent experiments (n = 3). Values with different superscripts within same column show significant differences ($P < 0.05$) between treatments according to the Duncan's multiple range test.

Authors' contribution

SMP designed experiments. MS performed experiments. MS, VPS and SMP analyzed data. MS, VPS and SMP wrote the manuscript.

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