



Nitrogen alleviates NaCl toxicity in maize seedlings by regulating photosynthetic activity and ROS homeostasis

Mamdouh M. Nemat Alla¹ · Nemat M. Hassan¹

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Abstract

Seven-day-old maize seedlings were subjected to NaCl (150 mM) without or with the presence of equimolar concentrations of urea, potassium nitrate or ammonium sulfate (at 60 kg N ha⁻¹) for the subsequent 15 days. The results show that NaCl significantly decreased photosynthetic activity parameters (Hill reaction, Pn, Ci and Gs), shoot fresh and dry weights, chlorophylls, protein, GSH and AsA. The activities of ALA-D, Rubisco, PEP-C, MDH, PPDK, SOD, CAT and APX were significantly inhibited. On the contrary, NaCl significantly elevated MDA, O₂⁻, H₂O₂, soluble sugars and proline. The application of the nitrogenous sources mostly ameliorated the photosynthetic activity synchronous with counterbalancing the decreases in growth, protein and pigments. The activities of ALA-D, Rubisco, PEP-C, MDH and PPDK enhanced remarkably with N applied under salinity. Furthermore, enhancements were detected in GSH and AsA contents and SOD, CAT and APX activities, which decreased free radicals, soluble sugars and proline. These effects reveal that maize was vulnerable to NaCl stress; however, N overcame the stress status and ameliorated the plant tolerance to salinity concomitant with repairing photosynthetic activity and maintaining higher antioxidants and ROS homeostasis; urea seemed to be the most efficient. Taken together, these findings conclude that N enhanced the capability of maize to tolerate NaCl via protecting photosynthetic apparatus for normal photochemical functioning and improving antioxidants and ROS homeostasis as effective salt tolerance mechanisms.

Keywords Antioxidants · Maize · NaCl · Nitrogen · Photosynthetic activity parameters · ROS homeostasis

Abbreviations

2,6-DCPIP	2,6-Dichlorophenolindophenol	Gs	Stomatal conductance
3-PGA	3-Phosphoglyceric acid	GSH	Glutathione
ALA	δ -Aminolevulinic acid	Hibitane	Chlorohexidine diacetate
ALA-D	δ -Aminolevulinic acid dehydratase	LSD	Least significant differences
ANOVA	Analysis of variance	MDA	Malondialdehyde
APX	Ascorbate peroxidase	MDH	Malate dehydrogenase
AsA	Ascorbic acid	Na-tricine	N-tris hydroxymethyl methyl glycine
CAT	Catalase	O ₂ ⁻	Superoxide radical
CDNB	1-Chloro-2,4-dinitrobenzene	OAA	Oxaloacetate
Ci	Sub-stomatal CO ₂ concentration	PEP	Phosphoenolpyruvate
CP	Creatine phosphate	PEP-C	Phosphoenolpyruvate carboxylase
CPase	Creatine phosphokinase	PGA1dDH	Glyceraldehydes-3-phosphate dehydrogenase
DTT	Dithiothreitol	PGK	Phosphoglycerate kinase
		Pn	Net photosynthetic rate
		PPDK	Pyruvate phosphate dikinase
		PVP	Polyvinyl pyrrolidone
		PVPP	Polyvinyl polypyrrolidone
		ROS	Reactive oxygen species
		Ru-1,5-P	Ribulose-1,5-bisphosphate
		Rubisco	Ru-1,5-P carboxylase/oxygenase

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✉ Mamdouh M. Nemat Alla
mamnematalla@du.edu.eg

¹ Botany Department, Faculty of Science, Damietta University, PO 34517 Kafr Saad, Egypt

SOD Superoxide dismutase
TCA Trichloroacetic acid

Introduction

Salt stress is deleterious for plant physiological and biochemical processes. It is toxic due to ions, oxidative and osmotic stresses, damaging of membranes, water deficiency, oxidative stress, imbalances of nutrients, metabolic dysfunction, denaturing cytosolic enzymes and alteration in growth regulators (Kong et al. 2017). Photosynthesis is the most important metabolic process in plants influenced by salt stress because of limitations in photosynthetic parameters (Pn, Ci and Gs). PSII is considered as crucial in plants photosynthesis (Perreault et al. 2011). In addition to these parameters, photosynthetic pigments and photosynthetic enzymes are fundamental for efficient photosynthesis; however, they are extremely sensitive to stress. Chlorophyll synthesis starts with the combination of two molecules of ALA (Wu et al. 2019) to form porphobilinogen by the action of ALA-D. Of the most important enzymes in C4 photosynthesis are PEP-C, MDH and PPDK besides Rubisco. The key enzyme in photosynthesis is Rubisco (Spreitzer 1993; Nemat Alla et al. 2011). It catalyzes the carboxylation of Ru-1,5-P forming 3-PGA in an irreversible reaction. PEP-C catalyzes the carboxylation of PEP to produce oxaloacetate which is reduced by NADP-MDH to malate with the production of CO₂ for Calvin cycle and pyruvate which undergoes conversion to PEP by the action of PPDK, regenerating thus the primary CO₂ acceptor.

Plants under salinity stress are affected by osmotic and oxidative stresses. Accumulation of soluble sugars and proline as active metabolites is claimed to be an effective tolerance mechanism to protect plants from osmotic stress by maintaining osmotic balance and increase the stability of macromolecules (Parida and Das 2005; Nemat Alla et al. 2016). Moreover, oxidative stress could result from over-generation of ROS which leads to disturbances in redox homeostasis (Chakraborty et al. 2019). They indicated that oxidative stress enhances peroxidation of membrane lipid, oxidation of protein, inhibition of enzymes and damage of DNA and RNA. However, a variety of antioxidants are developed in plants to balance the load of cellular ROS. The enzymatic antioxidants such as SOD, CAT and APX are important indices for the reflection of plant resistance to stress (Borella et al. 2019). Non-enzymatic metabolites, i.e., GSH and AsA, are associated with the balance of the cellular redox that could act as signals for the antioxidant mechanism regulation (Nemat Alla and Hassan 2014; Badran et al. 2015; Chakraborty et al. 2019).

Although plants have an antioxidant system to tolerate harsh conditions, this system might be insufficient in

sensitive species, so, external supporters are effective in stimulating plant growth and could support plants in tolerating stresses (Hassan et al. 2015; Perveen et al. 2018). N is very important for plant growth and development, so its exogenous application to plants could increase the ability of plants to withstand stress situations of salinity. It is integrated into several N-containing compounds and, moreover, it has important roles in regulating photosynthetic activity. So, the present work aims to investigate the effect of the external application of some nitrogenous sources (urea, potassium nitrate or ammonium sulfate) on modulating photosynthetic activity and ROS homeostasis in maize seedlings to tolerate NaCl stress.

Materials and methods

Conditions of growth

Maize (*Zea mays* L. Giza 2) grains were sterilized using 3% NaOCl for 10 min. After washing, the seeds were soaked for 8 h in water, then sown in perlite in 50 pots (35 cm in diameter, 10 seeds per pot) and allowed to establish for 7 days. The pots were distributed in five groups, the first to serve as control and the others for NaCl treatments (150 mM) alone or combined with equimolar concentrations of urea (46.5% N), potassium nitrate (13.8% N) or ammonium sulfate (21.2% N), each added at 60 kg N ha⁻¹. The pots were kept at 60% RH, 30 ± 2/15 ± 2 °C day/night, 16 h light and 300 μmol m⁻² s⁻¹ PPFD). Water was supplied daily. The experiment was repeated twice (100 pots). The collection of samples was carried out at the time of application (zero time) and at 5, 10 and 15 days after the treatment, washed thoroughly and shoots were separated, weighed then dried for determination of fresh and dry weights, respectively. The second leaves were used for the different analyses, fresh for photosynthetic activity determinations. For the other analyses, samples were frozen using liquid nitrogen.

Determination of photosynthetic activity

PSII activity was measured by the photoreduction rate of 2,6-DCPIP (Trebst 1972). Leaves were homogenized in Na-tricine (50 mM, pH 7.8) containing sucrose and magnesium chloride (300 and 3 mM, respectively). The pellets were collected by centrifugation and resuspended using Na tricine (1 mM, pH 7.8) containing NaCl and magnesium chloride (10 mM each) and absorbance was monitored at 600 nm. Photosynthetic gas-exchange variables (Pn, Ci and Gs) were determined using InfraRed Gas Analyzer in fully expanded leaves between 10 and 12 am. The conditions were set for determinations for CO₂ concentration

(at 360 ± 20 ppm), temperature (at 30 ± 2 °C), and relative humidity (at $60 \pm 4\%$).

Determination of protein and pigments

Protein was extracted from fresh tissue with chilled acetone. After filtration, residues were dried and the acetone powders were obtained (Harborne 1984). Extraction was completed from aliquots of powders using Tris–HCl (pH 9, 50 mM) and then centrifuged at $48,200 \times g$ for 15 min at 4 °C. Determination of protein content was carried out according to Bradford (1976). Chlorophyll contents were determined according to Metzner et al. (1965).

Determination of soluble sugars and proline

The extraction of soluble sugars was performed in 80% ethanol. An aliquot was mixed, reacted with anthrone reagent (8.6 mM anthrone in 80% H_2SO_4), heated, cooled in ice bath and the absorbance was measured at 623 nm according to Schlüter and Crawford (2001). The extraction of proline was carried out in 3% sulfosalicylic acid, then aliquots of the extracts were reacted with glacial acetic acid and acid ninhydrin and then absorbance was read at 520 nm according to Bates et al. (1973).

Determination of GSH and AsA

TCA (5%, w/v) containing 10 mM EDTA was used for the extraction of GSH. After centrifugation at $12,000 \times g$ for 15 min, aliquots were used for the assay of GSH using phosphate buffer (pH 6.8, 100 mM) with 10 mM EDTA, 1 mM CDNB and 1.0 U glutathione-S-transferase and incubated at 35 °C for 30 min, then absorbance was measured at 340 nm as described by Anderson and Gronwalds (1991). Phosphoric acid (62.5 mM) was used for the extraction of AsA. The extracts were centrifuged at $12,000 \times g$ for 20 min and then eluted with 4.5 mM H_2SO_4 using ion exclusion column. Folin–Ciocalteu reagent and 10% TCA were used for the assay of AsA (Mukherjee and Choudhuri 1983) and absorbance was measured at 760 nm.

Determination of MDA, O_2^- and H_2O_2

TCA (10%, w/v) was used for the extraction of MDA. After being centrifuged, MDA content was measured using 10% TCA containing 0.5% thiobarbituric acid and absorbance was read at 450, 532 and 600 nm. MDA content was determined from the equation: $6.45(A_{532} - A_{600}) - 0.56A_{450}$ (Sun et al. 2010). The extraction of O_2^- was carried out in 200 mM phosphate buffer (pH 7.2) with 1 mM diethyl dithiocarbamate, 3 mM potassium cyanide and 5 mM H_2O_2 . O_2^- was measured by determining the nitroblue tetrazolium

chloride reduction at 540 nm as described by Chaitanya and Naithani (1994). H_2O_2 was extracted in 0.1% TCA and the assay was performed in potassium phosphate buffer containing KI and absorbance was measured at 390 nm (Okuda et al. 1991).

Enzyme extraction and assays

The extraction of ALA-D was carried out with Tris–HCl (0.05 M, pH 9). The assay was conducted using Tris–HCl (50 mM, pH 7.0) and ALA (5 mg ml^{-1}). The quantity of porphobilinogen formed was measured at 555 nm as described by Mauzerall and Granick (1956) using the modified Ehrlich's reagent. Rubisco was extracted with Tris–HCl (20 mM, pH 8.0) containing 10 mM magnesium chloride, 10 mM sodium bicarbonate, 5 mM DTT, 1 mM EDTA, 0.002% Hixitane, and 1% PVP (Keys and Parry 1990). Hepes–KOH (50 mM, pH 7.5) with 10 mM DTT was used to extract PEPC, MDH and PPDK (Ashton et al. 1990). Bovine serum albumin, magnesium chloride and potassium phosphate were added during the extraction of PEP-C, while potassium phosphate was used for PPDK extraction. Rubisco activity was assayed in Hepes (50 mM, pH 7.8) containing 10 mM sodium bicarbonate, 0.66 mM Ru-1,5-P, 5 mM ATP, 20 mM magnesium chloride, 0.2 mM NADPH, 5 mM CP, 2.0 U CPase, 2.8 U 3-GAlDH and 2.0 U PGK kinase. Tris–HCl (25 mM, pH 8.0) was used for PEP-C activity assay in the presence of 5 mM magnesium chloride, 1 mM potassium bicarbonate, 2 mM DTT, 5 mM G-6-P, 5 mM PEP, 0.2 mM NADH and 2 U of MDH. Tricine–KOH (25 mM, pH 8.3) was used for MDH activity assay in the presence of 70 mM KCl, 1 mM EDTA, 1 mM DTT, 1 mM OAA and 0.2 mM NADPH. Hepes–KOH (25 mM, pH 8.0) was used for PPDK activity assay in the presence of 8 mM magnesium sulfate, 10 mM sodium bicarbonate, 5 mM ammonium sulfate, 10 mM DTT, 2 mM pyruvate, 1 mM G-6-P, 1 mM ATP, 2.5 mM potassium phosphate, 0.2 mM NADH, 0.5 U PEP-C and 2 U of MDH. The absorbance for all enzymes was read at 340 nm. The enzymatic antioxidants (SOD, CAT and APX) were extracted with 50 mM sodium phosphate buffer (pH 7.0) with 1 mM EDTA and 0.5% PVPP, then centrifuged at $13,000 \times g$. SOD activity was evaluated by measuring the inhibition of nitroblue tetrazolium photochemical reduction (Dhindsa et al. 1981). CAT activity was assayed as described by Beer and Sizer (1952) through the absorbance reduction of H_2O_2 at 240 nm. The activity of APX was assayed Nakano and Asada (1981) from recording absorption decrement at 290 nm due to ascorbate oxidation.

Statistical analysis

The pots of this work were arranged in a complete randomized block. The design consisted of 100 pots from

duplicate repetition of the experiment, five different treatments and 10 replications for each treatment. The values were calculated from six replicates as mean \pm SD. ANOVA was applied to full data, followed by LSD at $P \leq 0.05$.

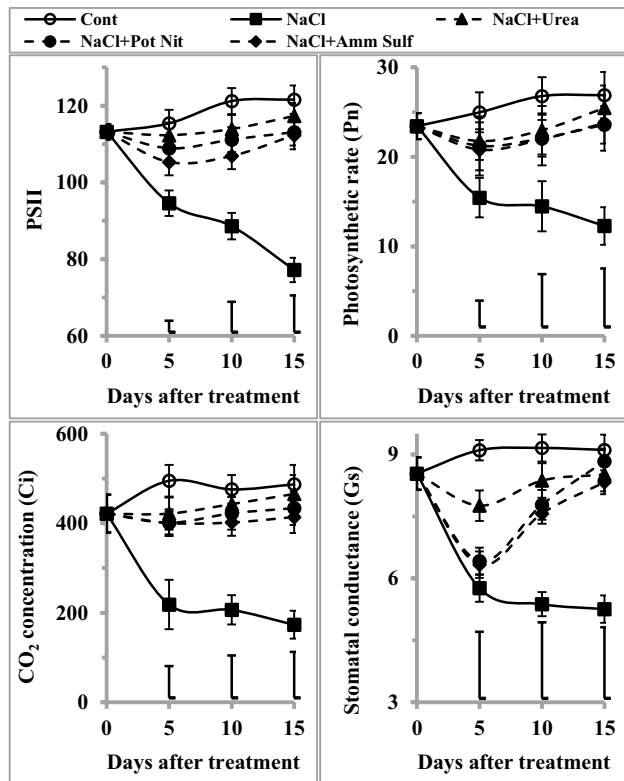
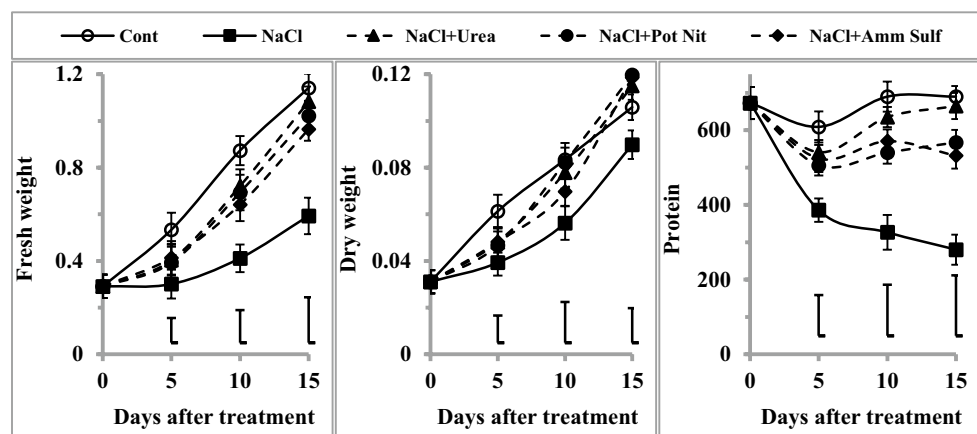


Fig. 1 Interaction of equimolar concentrations of the nitrogenous sources (urea, potassium nitrate or ammonium sulfate each added at 60 kg N ha⁻¹) on the parameters of photosynthetic activity (Hill reaction, PSII as $\mu\text{M DCPiP mg}^{-1}$ chlorophyll h⁻¹ and Pn, Ci, and Gs as mol m⁻² s⁻¹) in shoots of 7-day-old maize seedlings treated with 150 mM NaCl during the following 15 days. Mean values (\pm SD) were of six replicates, and LSD (vertical bars) was calculated at $P \leq 0.05$

Fig. 2 Interaction of equimolar concentrations of the nitrogenous sources (urea, potassium nitrate or ammonium sulfate each added at 60 kg N ha⁻¹) on fresh and dry weights (g per plant) and protein content (mg g⁻¹ DW) in shoots of 7-day-old maize seedlings treated with 150 mM NaCl during the following 15 days. Mean values (\pm SD) were of six replicates, and LSD (vertical bars) was calculated at $P \leq 0.05$



Results

Photosynthetic activity and growth parameters

The changes in photosynthetic activity parameters (Hill reaction, Pn, Ci, Gs) in 7-day-old maize seedlings throughout 15 days from application of 150 mM NaCl are depicted in Fig. 1. NaCl significantly declined the photosynthetic activity parameters in maize leaves throughout the experimental period in comparison with controls; an augmented decline was detected with the elapse of time. Due to NaCl treatment, the reduction was noted on the 15th day following treatment as 37% in Hill reaction, 54% in net photosynthesis rate, 64% in CO₂ concentration and 42% in stomatal conductance. Nonetheless, the application of equimolar concentrations of the nitrogenous sources (urea, potassium nitrate or ammonium sulfate) to salinity-treated seedlings greatly counterbalanced the NaCl-induced reductions in photosynthetic parameters; urea was in particular the most efficient. By the 15th day following treatment, the induced reductions in all parameters of the photosynthetic activity by NaCl were highly withdrawn following N application to become only 3–6% by urea, 3–13% by potassium nitrate or 7–15% by ammonium sulfate.

Fresh weight, dry weight and protein contents in maize shoots were also significantly decreased by NaCl treatment, and the magnitude of decrease by NaCl was augmented with the elapse of time (Fig. 2). Fresh weight was more affected on the 15th day from treatment than dry weight (48 and 15%, respectively), while the reduction in protein content was about 34%. However, the application of the nitrogenous sources induced retractions in the effects of NaCl; urea was more efficient than potassium nitrate or ammonium sulfate. NaCl-induced decreases in fresh weight and dry weight were retracted on the 15th day from application of NaCl to become, respectively, 5 and 9% by urea, 11 and 12% by potassium nitrate or 15 and 12% by ammonium sulfate;

however, slight increases were detected in the protein content of NaCl-treated seedlings by urea, potassium nitrate or ammonium sulfate (10, 4 and 3%, respectively).

Chlorophyll and photosynthetic enzymes

Similarly, NaCl treatment led to significant reductions in chlorophyll contents in relation to the untreated controls, and the magnitude of reductions became about 25 and 21% in chlorophyll *a* and chlorophyll *b*, respectively, by the 15th day following treatment (Fig. 3). The ratio of chlorophyll *a/b* exhibited the lowest values in NaCl-treated seedlings all over the experiment, but the highest ones were detected in the untreated control. At the same time, significant inhibitions were detected in ALA-D activity in maize leaves by NaCl treatment throughout the entire experimental period, and the magnitudes of inhibition was 44% on the 15th day of treatment. Nevertheless, these decreases were nullified upon the application of urea, potassium nitrate or ammonium sulfate. Moreover, the application of the nitrogenous sources resulted in

moderate values of chlorophyll *a/b* ratios between NaCl-treated and the untreated control. The nitrogenous sources lowered the effects of NaCl to become on the 15th day following treatment only 6, 10 and 11%, respectively, in chlorophyll *a*, 5, 7 and 8% in chlorophyll *b* or 4, 11 and 12% in ALA-D activity.

Figure 4 shows that NaCl treatment significantly inhibited the activities of Rubisco, PEP-C, MDH and PPDK throughout the entire experiment as compared to controls: the effect was fast and high for Rubisco and PEP-C, but steady for MDH and PPDK; the magnitudes of inhibition in Rubisco, PEP-C, MDH and PPDK on the 15th day from treatment was noted as 60, 54, 48 and 35%, respectively. The application of the nitrogenous sources, particularly urea, greatly counterbalanced the NaCl-induced inhibitions. By the 15th day following treatment, the inhibitions in the enzyme activities by NaCl were highly withdrawn by urea, potassium nitrate or ammonium sulfate to become only 11, 26 and 20% in Rubisco, 27, 22 and 23% in PEP-C, 7, 16 and 17% in MDH and 7, 18 and 16% in PPDK.

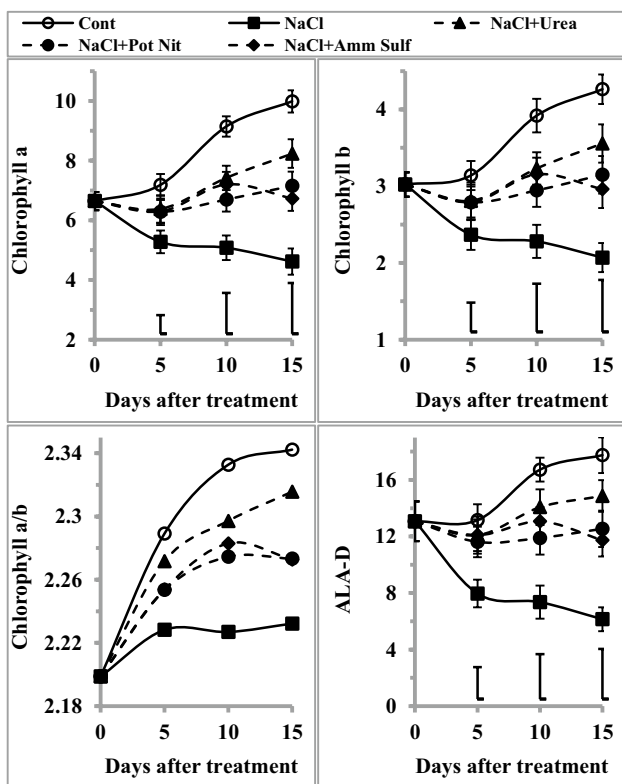


Fig. 3 Interaction of equimolar concentrations of the nitrogenous sources (urea, potassium nitrate or ammonium sulfate each added at 60 kg N ha⁻¹) on chlorophyll *a* and *b* contents (mg g⁻¹ DW), their ratio and ALA-D activity (mg porphobilinogen released g⁻¹ DW h⁻¹) in shoots of 7-day-old maize seedlings treated with 150 mM NaCl during the following 15 days. Mean values (\pm SD) were of six replicates, and LSD (vertical bars) was calculated at $P \leq 0.05$

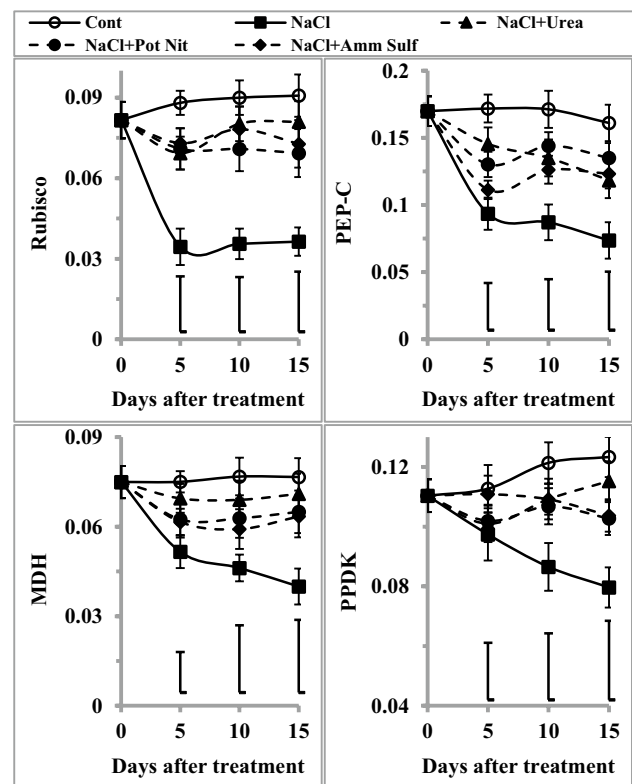


Fig. 4 Interaction of equimolar concentrations of the nitrogenous sources (urea, potassium nitrate or ammonium sulfate each added at 60 kg N ha⁻¹) on Rubisco, PEP-C, MDH and PPDK activities (decrease in absorbance mg⁻¹ protein min⁻¹) in 7-day-old maize shoots treated with 150 mM NaCl during the following 15 days. Mean values (\pm SD) were of six replicates, and LSD (vertical bars) was calculated at $P \leq 0.05$

Osmoprotectants, antioxidants and ROS

Soluble sugars and proline contents were significantly elevated by NaCl during the whole experiment as compared to controls; however, GSH and AsA contents were significantly decreased (Fig. 5). The magnitude of elevation

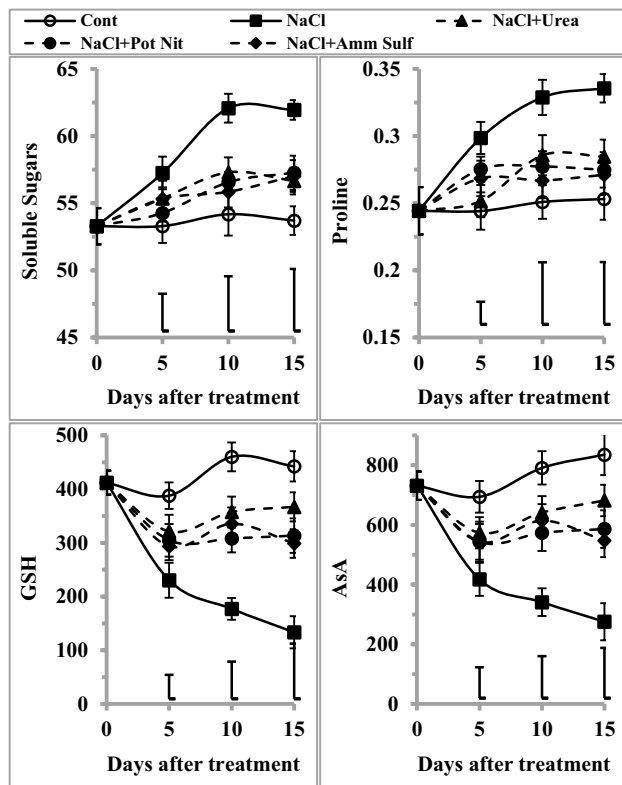
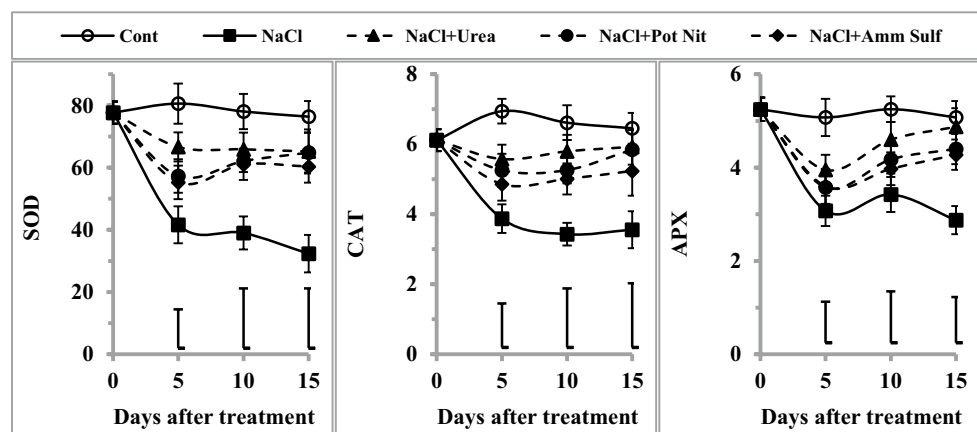


Fig. 5 Interaction of equimolar concentrations of the nitrogenous sources (urea, potassium nitrate or ammonium sulfate each added at 60 kg N ha⁻¹) on soluble sugars, proline contents (mg g⁻¹ dry weight), GSH and AsA contents (μg g⁻¹ dry weight) in shoots of 7-day-old maize seedlings treated with 150 mM NaCl during the following 15 days. Mean values (±SD) were of six replicates, and LSD (vertical bars) was calculated at $P \leq 0.05$

Fig. 6 Interaction of equimolar concentrations of the nitrogenous sources (urea, potassium nitrate or ammonium sulfate each added at 60 kg N ha⁻¹) on SOD, CAT and APX activities (Units mg⁻¹ protein) in 7-day-old maize shoots treated with 150 mM NaCl during the following 15 days. Mean values (±SD) were of six replicates, and LSD (vertical bars) was calculated at $P \leq 0.05$

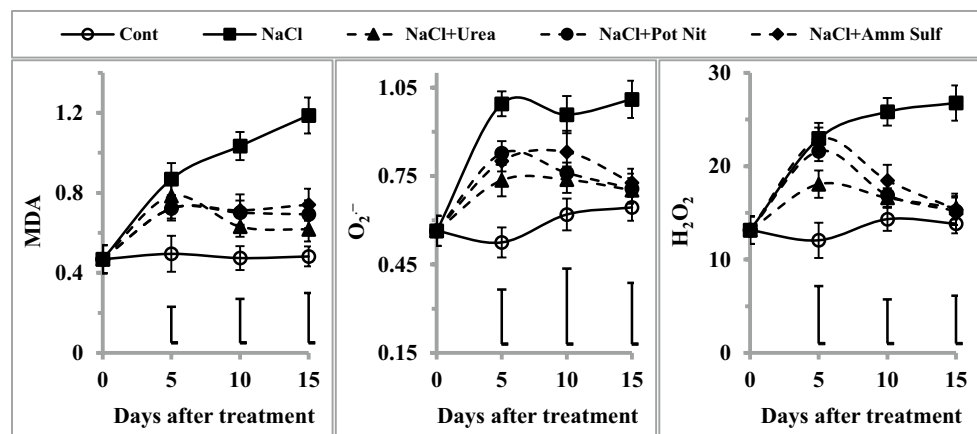


of soluble sugars and proline reached about 22 and 40%, respectively, by the 15th day from treatment, while the drop of GSH or AsA reached about 51 or 45%, respectively. Anyway, the use of the nitrogenous sources greatly alleviated the rises in soluble sugars and proline as well as the decrease in GSH and AsA such that on the 15th day from treatment, the increases in soluble sugars retracted to only 9, 5 and 3% by urea, potassium nitrate or ammonium sulfate, respectively, while the increases in proline retracted to 16, 6 and 4%. However, the decreases in GSH were 5, 11 and 10%, while under the same conditions the decreases in AsA were 7, 12 and 13%, respectively.

The activities of the antioxidant enzymes were remarkably inhibited following NaCl treatment; these inhibitions reached about 58, 45 and 43% in SOD, CAT and APX, respectively, by the 15th day of NaCl application (Fig. 6). These inhibitions in activities were markedly retracted upon the application of urea, potassium nitrate or ammonium sulfate to become, respectively, on the 15th day of treatment only 15, 18 and 21%, respectively. in SOD, 8, 10 and 19% in CAT or 4, 14 and 16% in APX.

On the contrary, the application of NaCl induced significant elevation in MDA, O₂⁻ and H₂O₂ contents throughout the experimental period (Fig. 7). The elevation was greater in MDA and H₂O₂ (146 and 130%, respectively, on the 15th day following treatment) relative to O₂⁻ (87%). The increments of these contents were greatly lowered upon the application of the nitrogenous sources such that the magnitude of increases became by urea, potassium nitrate ammonium only 28, 34 and 54% in MDA, 13, 23 and 36% in O₂⁻ or 17, 22 and 34% in H₂O₂.

Fig. 7 Interaction of equimolar concentrations of the nitrogenous sources (urea, potassium nitrate or ammonium sulfate each added at 60 kg N ha⁻¹) on the contents of MDA (absorbance decrease min⁻¹ mg⁻¹ protein), O₂⁻ (Δ540 g⁻¹ DW min⁻¹) and H₂O₂ (μ mol g⁻¹ DW) in shoots of 7-day-old maize seedlings treated with 150 mM NaCl during the following 15 days. Mean values (±SD) were of six replicates, and LSD (vertical bars) was calculated at *P* ≤ 0.05



Discussion

Photosynthetic activity and growth parameters

Salinity affects plants by various effects, including growth reductions, ion toxicity, energy waste, reduced carbon fixation and shortage of photosynthetic activities (Kong et al. 2017). Under salinity stress, salts can reach excessive levels in the apoplast that would lead to cell dehydration, they may accumulate in the cytoplasm with a consequent inhibition in enzymes or may be increased in the chloroplast that causes toxicity to photosynthesis (Munns and Tester 2008). The present study showed that the decrease detected in photosynthetic activity, growth parameters and protein of NaCl-treated seedling may result from the deleterious influences of ions and to the N-metabolism reduction. Nonetheless, these effects were mostly counterbalanced by the application of N sources, most effectively urea since N has important roles under saline conditions both in nutrition and in osmosis.

The significant reduction in photosynthesis induced by salinity might be due to stomatal and/or non-stomatal factors. Stomatal factors such as stomatal closure could limit gas-exchange parameters in leaves, whereas non-stomatal factors are mostly cell membrane damage and inhibition in the activity of Rubisco (Lang et al. 2018). Such response in the efficacy of photosynthesis toward salinity was, indeed, detected in maize in the present results. In fact, NaCl depressed Hill reaction, Pn, Ci and Gs in maize leaves. So, stomatal resistance became higher and gas exchange became lower than normal with consequences of retardations in the photosynthetic machinery. Jedmowski et al. (2013) indicated that stomatal closure could raise CO₂ diffusion resistance, which could depress the photosynthetic rate with a consequent drop in photosynthesis. So, the decreased intercellular CO₂ concentration and stomatal conductance, in the present study, indicate that maize leaves underwent stomatal closure by NaCl with a consequent reduction in the CO₂

concentration diffusing into leaves causing reduction in photosynthetic rate.

The impacts on photosynthetic parameters induced in NaCl-stressed maize seedlings were reduced with the application of the nitrogenous sources. As compared to the stressed seedlings, urea in the same conditions elevated Hill reaction, net photosynthesis rate, CO₂ concentration and stomatal conductance to more than 1.5-, 2.0-, 2.5- and 1.6-fold. Similar elevation—but to a lower extent—were also detected by potassium nitrate or ammonium sulfate. These findings conclude that N supplementation protected the photosynthetic apparatus and mitigated the salinity-induced damage. In general, N supplies nutrition for plant growth and so could minimize the salinity-induced nutrient deficiency. As shown in the results, urea was the most efficient.

In fact, urea is an important N form, because it is considered as the major fertilizer in addition to its importance among the plant metabolites. So, appropriate levels could ameliorate several reactions in the physiology and biochemistry of plants under stress conditions, maintain relative water content and alleviate stress-induced damage. The changes in photosynthetic parameters by NaCl and the recovery by N application were in conformity with growth parameter changes. So, N as an exogenous supporter was needed for maize to withstand salinity stress. Tolerance is a highly desirable trait in crops; the salt tolerance/resistance is, however, slight in these crops. Plants have an antioxidant system to tolerate harsh conditions; nonetheless, this system might be insufficient in sensitive species under abiotic stresses. So, several strategies have been performed to alleviate plant growth reduction through mitigating the adverse effects of stress (Wu et al. 2019).

Chlorophyll and photosynthetic enzymes

The efficiency of photosynthesis depends, besides the photosynthetic parameters, on the photosynthetic pigments and enzymes; they could be decreased by salinity and would

decline the photosynthetic activities. Chlorophylls are essential for photosynthesis and, moreover, they are markers for plant photosynthetic efficiency at varied situations. ALA-D enzyme is the key for the biosynthesis of chlorophylls. The present results show that NaCl significantly decreased chlorophylls and ALA-D activity. In confirmation, the degradation of pigments by salt stress reflected vulnerability of photosystems to salt stress (Mazumdar et al. 2019). However, application of nitrogenous sources increased protein for enzyme activities and stimulated ALA-D for chlorophyll biosynthesis. This improvement would compensate for the influence of PSII by the effects of excessive energy of excitation as indicated by Gururani et al. (2015). The enhancement was most pronounced with urea. In addition, the ratio of chlorophyll *a/b* was negatively affected by NaCl, indicating the stress impact on the photoinhibition risks of photosystem composition; however, N rendered these values close to normal. As indicated, N is essential for the structure of chlorophylls, so an exogenous supply would provide a sufficient amount of N to promote chlorophyll biosynthesis. In this account, Kumawat and Mahla (2015) concluded that urea provided more appropriate substrates for pigment biosynthesis and stimulated photosynthesis, enhanced leaf dry matter production and promoted plant growth. Therefore, urea could enhance photosynthetic pigment contents which exhibit a positive relationship with photosynthetic parameters. So, the inefficient photosynthesis in salt-stressed plants could be regarded mainly due to the reduced pigment contents; however, N induced amelioration in photosynthesis possibly due to enhancing pigment biosynthesis and/or to an elevation in the rigidity of cell wall.

In integration with photosynthetic capacity in maize, the photosynthetic pathway in C4 plants involved some additional enzymatic reactions that precede the Calvin cycle. Therefore, Rubisco catalyzes the carboxylation of Ru-1,5-P in the Calvin cycle for maintaining the rate of carbon fixation. However, these additional special reactions convert PEP to OAA by a carboxylation reaction stimulated by PEP-C, reducing OAA to malate and then pyruvate by MDH, and phosphorylating pyruvate to PEP by PPDK for the generation of the first acceptor of CO₂ in C4. The activities of these enzymes in addition to Rubisco, in the present results, were significantly inhibited by NaCl, causing further retardation of the photosynthetic machinery. Due to the stomatal closure by NaCl, the inhibition in PEP-C would retard OAA formation for MDH with a consequent decline both in CO₂ release for Rubisco and Calvin cycle and in pyruvate for PPDK. In confirmation, significant inhibitions were also detected in PPDK, PEP-C and MDH in maize due to different stress conditions (Nemat Alla and Hassan 1998; Hassan et al. 2008). So, an inhibition in these enzymatic activities could explicate the decreased rate of photosynthetic activity. However, these inhibitions were markedly alleviated by N

sources, concomitant with a repair in the photosynthetic process. The enzyme activity enhancement was synchronized with the improvement in CO₂ concentration and stomatal conductance that would subsequently ameliorate photosynthetic rate. The results clearly indicate the coincidence of the changes in enzyme activities with the changes in photosynthetic parameters and also with chlorophyll and growth parameters.

Osmoprotectants, antioxidants and ROS

Under abiotic stress conditions, plants accumulate considerable organic solutes to protect the functioning of normal metabolic processes, and also other processes for the metabolism of adaptation to depress ROS extra production. Soluble sugars and proline are low-molecular-weight compatible solutes that avert the harmful effects of stress. Hayat et al. (2012) indicated that these osmoprotectants lower inhibitions induced in enzyme activities by ions, raise the enzyme thermal stability and foreclose enzyme complex dissociation. On the other hand, Szabados and Savouré (2010) reported that proline could regulate the function of mitochondria; moreover, it acts in the redox balancing modulation. Koenigshofer and Loeppert (2019) concluded that plants produce proline to protect the different meristematic tissues. The present results are in conformity with these findings; there were increased accumulations in soluble sugars and proline in maize shoots by NaCl, while the presence of nitrogenous sources, particularly urea, overcame these accumulations. These dramatic changes could be due to changes in their metabolic processes and to disorder in their transportation. Soluble sugars and proline act likely in counterbalancing salinity-induced rises of osmotic potential and protecting cell components through elimination of ROS. However, the retractions in their values by N application indicate that N relieved maize from salinity stress, and the relief was most evident with urea.

On the other hand, the overproduction of MDA, H₂O₂ and O₂⁻ induces oxidative damage in plant cells and disturbs redox homeostasis (Nemat Alla et al. 2007; Chakraborty et al. 2019). ROS are generated in plant seedlings comprising important requirements in signaling; however, their ranges should remain suitable, and salinity adversely affected this scenario. Plants have to overcome these imbalances in metabolism through the ROS detoxification machinery. In the present results, the increments of ROS contents in NaCl-treated maize seedlings were greatly lowered upon the application of the nitrogenous sources, indicating the induction of NaCl-induced oxidative stress in treated samples while N stimulated the relief. On the contrary, NaCl greatly dropped SOD, CAT and APX activities; a similar drop was also detected for GSH and AsA contents; however, more inductions were detected following N supplementation.

These results affirm that N application highly counterbalanced the diminutions in antioxidants and the elevations in ROS confirming that N enforced maize to enhance the enzymatic and nonenzymatic antioxidants to tackle with oxidative damages. The ameliorations in these antioxidants along with the retraction in the oxidative stress parameters could conclude that N, particularly urea, improved antioxidants and ROS homeostasis as effective salt tolerance mechanisms. Urea could increase antioxidant activities by adjusting osmosis and activating the antioxidants for alleviation of oxidative nuisance and scavenging of ROS (Kaya et al. 2015). Moreover, Zhang et al. (2015) concluded that urea remarkably elevated N and nitric oxide which could reduce the generation of ROS, stimulate the SOD, CAT and POD activities, and enhance photosynthetic pigments. So, improving antioxidants, ROS homeostasis and osmoregulation were generally synchronized with the ameliorations in photosynthetic activity and growth parameters. These findings confirm that N could increase maize tolerance to NaCl through effectors of stress adaptation that mediate osmoregulation, antioxidants, ROS homeostasis and photosynthetic activity.

Conclusion

The significant decrease in photosynthetic activity parameters (Hill reaction, Pn, Ci and Gs) of maize seedlings by NaCl was synchronized with remarkable decreases in fresh and dry weights, protein, chlorophylls, GSH and AsA, along with great depressions in ALA-D, Rubisco, PEP-C, MDH, PPDK, SOD, CAT and APX activities; however, there were significant stimulations in MDA, O_2^- , H_2O_2 , soluble sugars and proline. The reduced growth might be due to low availability of N under adverse conditions, since vital processes in plants need N for biological systems. The depressions in photosynthetic activity and enzymes and in antioxidants concomitantly with the accumulated ROS could reveal that maize was vulnerable to suffer from NaCl stress. On the contrary, this study clearly showed that N effectively mitigated salt stress impacts. N stimulated growth and reduced NaCl negative effects on the photosynthetic apparatus and on the antioxidant system and ROS homeostasis. These improvements indicate a repair in photosynthetic activity and antioxidant system by N; urea seemed the most efficient. So, the present findings conclude that N increased maize tolerance to NaCl through protecting the photosynthetic apparatus and improving antioxidants and ROS homeostasis.

Author contribution statement MMN and NMH have the same contribution toward the experiment design, attainment of data, analysis and performance of the experiment and prearranging of the manuscript. The ultimate manuscript was read and approved by MMN and NMH.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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