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Nitrogen Form Alters Hormonal Balance in Salt-treated Tomato (*Solanum lycopersicum* L.)

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Abstract Mixed nitrate/ammonium fertilization can partially alleviate the negative effects of salinity on growth of some plant species compared to all-nitrate or all-ammonium fertilization. To gain insights about the mechanisms involved, tomato (*Solanum lycopersicum* L. cv Moneymaker) plants were grown hydroponically for 3 weeks with two NO_3^-/NH_4^+ fertilization regimes (6/0.5 and 5/1.5; $N_{total} = 6.5$ mM) in the absence (control) or presence of salt stress (100 mM NaCl). Ammonium enrichment had no effect on growth and other parameters under control conditions. Under salinity, however, ammonium enrichment improved shoot and root biomass by 20% and maintained leaf PSII efficiency close to control levels. These changes were related to higher leaf K^+, NO_3^-, and NH_4^+ concentrations and activities of the N-assimilatory enzymes glutamate synthase (GOGAT) and glutamine synthase (GS) in the leaves. Ammonium enrichment also attenuated the salt-induced increase in leaf abscisic acid (ABA) concentration and decrease in leaf concentrations of indole 3-acetic acid (IAA) and the cytokinins *trans*-zeatin (*t*Z) and *trans*-zeatin riboside (*t*ZR). Enhanced cytokinin status was probably due to maintenance of root-to-shoot cytokinin transport and decreased leaf induction of the cytokinin-degrading enzyme cytokinin oxidase/dehydrogenase (CKX) under ammonium-enriched conditions. It is concluded that nitrogen form modifies salinity-induced physiological responses and that these modifications are associated with changes in plant hormone status.

Keywords Abscisic acid · Ammonium · Cytokinin · Indole-3-acetic acid · Nitrate · Nitrogen metabolism · Plant hormones · Salt stress · Sodium chloride · Tomato (*Solanum lycopersicum* L.) · *trans*-Zeatin · *trans*-Zeatin riboside

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Introduction

Nitrogen is required in large quantities by plants and is usually taken up in the form of ammonium (NH_4^+) and nitrate (NO_3^-). Ammonium in plants originates from direct uptake, nitrate reduction, deamination of nitrogenous compounds, or the photorespiratory nitrogen cycle (Lasa and others 2002). Ammonium is assimilated in plants into amino acids via the glutamine synthase (GS)/glutamate synthase (GOGAT) cycle or via the glutamate dehydrogenase (GDH) alternative ammonium assimilation pathway (Frechilla and others 2002; Lasa and others 2002). In the GS/GOGAT cycle, GS catalyzes the conversion of ammonium and glutamate to glutamine. Glutamate synthase (GOGAT) then catalyzes the transfer of the amide group of glutamine to 2-oxoglutarate and produces two molecules of glutamate, one of them being recycled as a substrate for the GS reaction. Glutamate dehydrogenase (GDH) catalyzes the transformation of α-ketoglutarate into glutamate, although it is considered to exhibit a higher K_m for ammonium than GS (Miflin and Habash 2002).

Compared with NO_3^- nutrition, mixed nitrogen nutrition ($NH_4^+ + NO_3^-$) often improves growth and yield in several cultivated plant species (Smiciklas and Below 1992). Although the physiological processes that are altered in plants grown with both N forms have yet to be fully understood, factors associated with reproductive development and a modified partitioning of assimilate have been implicated (Smiciklas and Below 1992). Growth inhibition induced by NH_4^+ as a sole N form is associated with ionic imbalances, uncoupling energy production, and energy cost of futile NH_4^+ cycling in roots (Britto and Kronzuker 2006), but it is also associated with a decrease in *trans*-zeatin *(t*Z) and *trans*-zeatin riboside (*t*ZR) concentrations in the xylem sap (Smiciklas and Below 1992 and references therein cited). Previous works have also shown that N status (Dodd and others 2004; Sakakibara and others 2006) and N form (Walch-Liu and others 2000) can alter cytokinin metabolism and transport in plants. Cytokinin supply is thus an important factor that could be related to enhanced growth and reproductive development induced by mixed $NH_4^+ + NO_3^-$ nutrition.

Salinity limits crop growth by inhibiting cell division and cell expansion rates during leaf growth, delaying leaf emergence, decreasing leaf photosynthesis, and accelerating leaf senescence (Munns 2002). It is commonly assumed that salt stress induces two consecutive constraints in plant tissues: the first a direct consequence of salt-induced osmotic stress whereas the second one results from toxic ion accumulation (Munns 2002). The mechanisms that downregulate leaf growth and shoot development during the osmotic phase of salt stress remain unknown. It has been hypothesized that leaf growth inhibition could be regulated by hormones or their precursors, because decreased leaf growth rate occurs independent of carbohydrate supply, water status, nutrient deficiency, and ion toxicity (see Munns and Tester 2008; Pérez-Alfocea and others 2010 for a review). Recent work showed that during the early (3 weeks) application of high salinity (100 mM NaCl) to tomato (*Solanum lycopersicum* L.), a decrease in leaf cytokinin (CK) concentration (Z + ZR) was temporally correlated with both salt-induced leaf senescence (Ghanem and others 2008) and shoot growth impairment (Albacete and others 2008), whereas a rootstock-mediated increase in leaf xylem *t*Z concentration was related to improved salt tolerance in tomato (Albacete and others 2009).

The response of plant growth and ammonium assimilation enzymes to nitrogen fertilization under saline and nonsaline conditions varies according to whether the nitrogen is supplied as nitrate or ammonium and also depends on plant species (Misra and Dwivedi 1990; Sagi and others 1998; Flores and others 2001; Kant and others 2007). Growth of many plants is impaired when nitrogen is applied solely as NH_4^+ (Gerendas and others 1997; Siddiqi and others 2002). However, sole application of NO_3^- under salinity stress may also result in nitrogen deficiency due to decreased nitrate uptake as a consequence of NO_3^-/Cl^- antagonism at the transport level (Pérez-Alfocea and others 1993; Botella and others 1994). Several plant species show enhanced growth under salt stress conditions when provided with both nitrate and ammonium compared to growth on only nitrate or ammonium (Siddiqi and others 2002; Kant and Kafkafi 2003). In tomato, salinity (60 mM NaCl for 2 weeks) decreased plant growth by 25% when plants were grown on nitrate alone, whereas no significant decrease occurred in the presence of a 6/1 NO_3^-/NH_4^+ ratio (Flores and others 2001). The underlying physiological mechanisms allowing such growth maintenance remain unknown. Interpretation of the beneficial effects of combined nitrate/ammonium nutrition on plant responses to salt stress has generally implicated changes in the ammonium assimilation pathways and ionic accumulation (Sandoval-Villa and others 1999; Flores and others 2001; Kant and others 2007), but the major groups of plant growth regulators have paradoxically been neglected. Recently, a beneficial impact of a mixed nitrogen treatment on the growth of tomato was suggested to result from a higher zeatin riboside (ZR) concentration in the xylem sap (Lu and others 2009), but these authors did not consider the impact of salinity on ammonium assimilation pathways and ionic accumulation. Besides cytokinins, salt stress also influences abscisic acid, ethylene, and auxin synthesis and translocation in plants (Ghanem and others 2008; Albacete and others 2009), but the impact of N form on these growth regulators requires further clarification.

The present work was therefore undertaken to test the following hypotheses: (1) nitrogen form may influence

plant growth and physiological behavior under salt stress through a direct impact on plant hormone status, and (2) nitrogen impact on both growth and hormonal content may differ in the presence and the absence of salt stress. Accordingly, tomato was grown under high-salinity stress (100 mM NaCl for 3 weeks) under two different NO_3^-/NH_4^+ treatments (half-strength Hoagland's solution, 6/0.5, and ammonium-enriched solution, 5/1.5), and the endogenous levels of four major plant hormones (abscisic acid, the auxin indole acetic acid, and the cytokinins zeatin and zeatin riboside) were analyzed in leaves, roots, and xylem sap. Temporal changes in ionic, biochemical, and hormonal variables were related to growth, stomatal conductance, and chlorophyll fluorescence to determine whether nitrogen form altered salt tolerance by regulating these physiological processes.

Materials and Methods

Plant Material and Culture Conditions

Seeds of tomato (*Solanum lycopersicum* L. cv. Moneymaker) were obtained from the Tomato Genetics Resource Centre (TGRC, University of California-Davis, CA, USA). Seeds were sown in trays filled with a perlite-vermiculite mix (1:3 v/v proportion) moistened regularly with half-strength Hoagland nutrient solution. Fourteen days after sowing, the substrate was gently washed from the roots and seedlings placed in a growth chamber on polyvinyl chloride plates floating on two types of aerated half-strength Hoagland solutions that differed in NO_3^-/NH_4^+ ratios (either 6/0.5 or 5/1.5) at 6.5 mM total nitrogen. Both solutions also contained the following chemicals (in mM): 5 KNO_3, 1 $NH_4H_2PO_4$, 0.5 $MgSO_4$, 5.5 $Ca(NO_3)_2$, and (in μM) 25 KCl, 10 H_3BO_3, 1 $MnSO_4$, 1 $ZnSO_4$, 0.25 $CuSO_4$, 10 Na_2MoO_4, and 1.87 mg L^{-1} Fe-EDDHA. Solutions were renewed every week, refilled every 2 days, and their pH adjusted daily to 5.5–6 using 5 M KOH.

Plants were grown in a growth chamber under a 16-h photoperiod. The air temperature ranged from 25 to 28°C during the day and from 17 to 18°C during the night. Relative humidity was maintained at 70 ± 5% during the night and at 50 ± 5% during the day. Light intensity at the top of the canopy was around 265 μmol m^{-2} s^{-1} (PPFD). After 4 days of acclimation in control conditions (18 days after sowing), the seedlings were exposed to 0 (control) or 100 mM NaCl added to the nutrient solution. Three replications with 8 plants per replication and salt treatment were used. An actively growing leaf, present at the moment that salt stress was applied (identified as leaf number 4, numbering from the base of the plant) was tagged for subsequent growth measurements, senescence monitoring, and harvest for biochemical determinations. Xylem sap was obtained from 3 plants per treatment after severing the shoot about 2–3 cm above the root and by applying pressure (−0.5 MPa for control plants and about −0.9 MPa for stressed ones) to the root system with a Scholander pressure chamber (Pérez-Alfocea and others 2000).

Chlorophyll Fluorescence and Stomatal Conductance

Modulated chlorophyll fluorescence was measured in tagged and dark-adapted (30 min) leaves in 6–10 plants per treatment using a chlorophyll fluorometer OS-30 (Opti-Sciences, Herts, UK), with an excitation source intensity of 3000 μmol m^{-2} s^{-1}. The minimal fluorescence intensity (F_0) in a dark-adapted state was measured in the presence of a background far-red light to favor rapid oxidation of intersystem electron carriers. The maximal fluorescence intensities in the dark-adapted state (F_m) and after adaptation to white actinic light (F_m') were measured by 0.8-s saturating pulses (3000 μmol m^{-2} s^{-1}). After the F_m' measurement, the actinic light (400 μmol m^{-2} s^{-1}) was switched off, and the far-red light was applied for 3 s to measure the minimal fluorescence intensity in the light-adapted state (F_0'). The maximum quantum yield of open photosystem II (PSII) (F_v/F_m) was calculated as $(F_m - F_0)/F_m$.

Leaf stomatal conductance (g_s) was determined at 10:0 a.m. on the abaxial surface of leaf 4 on 10 plants per treatment using an AP4 diffusion porometer (Delta-T Devices Ltd., Cambridge, UK).

Ion Concentrations

For K^+ and Na^+ quantification, leaf and root tissues were frozen with liquid nitrogen. After thawing, the samples were centrifuged for 10 min at 10,000 *g* and then for 5 min at 20,000 *g* to obtain bulk tissue sap. Necessary dilutions were performed to measure K^+ and Na^+ concentrations, determined by using a Shimadzu AA-680 atomic absorption spectrophotometer (Shimadzu Ltd, Kyoto, Japan). All measurements were performed in three replicates. Nitrate concentrations were determined in water extracts of the freeze-dried root and leaf samples using a modified salicylic acid method (nitration of salicylic acid) as described by Cataldo and others (1974), whereas NH_4^+ concentrations were determined by coupled enzymatic analysis after conversion of NH_4^+ and 2-oxoglutarate into L-glutamate and determination of NADH at 340 nm using the Roche Diagnostic Urea/Ammonia Kit (Roche Diagnostics, Mannheim, Germany).

Hormone Extraction and Analysis

Hormones were analyzed as previously described (Albacete and others 2008; Ghanem and others 2008). Cytokinins

[zeatin (Z) and zeatin riboside (ZR)], indole-3-acetic acid (IAA), and abscisic acid (ABA) were extracted and purified according to the method of Dobrev and Kaminek (2002). One gram of fresh plant material (leaf or root) was homogenized in liquid nitrogen and placed in 5 ml of cold (−20°C) extraction mixture of methanol/water/formic acid (15/4/1 v/v, pH 2.5). After overnight extraction at −20°C, solids were separated by centrifugation (20,000 *g*, 15 min) and re-extracted for 30 min in an additional 5 ml of the same extraction solution. Pooled supernatants were passed through a Sep-Pak Plus †C18 cartridge (SepPak Plus, Waters, Milford, MA, USA) to remove interfering lipids and plant pigments and evaporated to dryness. The residue was dissolved in 5 ml of 1 M formic acid and loaded on an Oasis MCX mixed mode (cation-exchange and reverse phase) column (150 mg, Waters) preconditioned with 5 ml of methanol followed by 5 ml of 1 M formic acid. To separate different CK forms (nucleotides, bases, ribosides, and glucosides) from IAA and ABA, the column was washed and eluted stepwise with different appropriate solutions as indicated in Dobrev and Kaminek (2002). Abscisic acid and IAA were analyzed in the same fraction. After each solvent was passed through the columns, they were purged briefly with air. Solvents were evaporated at 40°C under vacuum. Samples were then dissolved in a water/acetonitrile/formic acid (94.9:5:0.1 v/v) mixture for HPLC/MS analysis. Analyses were carried out on a HPLC/MS system consisting of an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) equipped with a μ-wellplate autosampler and a capillary pump and connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray (ESI) interface. Prior to injection, 100 µl of each fraction extracted from tissues or a similar volume of xylem sap was filtered through 13-mm-diameter Millex filters with a 0.22-µm-pore nylon membrane (Millipore, Bedford, MA, USA). For each sample, 8 µl dissolved in mobile phase A was injected onto a Zorbax SB-C18 HPLC column (5 µm, 150 × 0.5 mm, Agilent Technologies), maintained at 40°C, and eluted at a flow rate of 10 µl min^{-1}. Mobile phase A, consisting of water/acetonitrile/formic acid (94.9:5:0.1), and mobile phase B, consisting of water/acetonitrile/formic acid (10:89.9:0.1), were used for the chromatographic separation. The elution program maintained 100% A for 5 min, then a linear gradient from 0 to 6% B in 10 min, followed by another linear gradient from 6 to 100% B in 5 min, and finally 100% B maintained for another 5 min. The column was equilibrated with the starting composition of the mobile phase for 30 min before each analytical run. The UV chromatogram was recorded at 280 nm with a DAD module (Agilent Technologies). The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V and a scan speed of 22,000 (*m*/*z*)/s from 50 to 500 *m*/*z*. The nebulizer gas (He) pressure was set to 30 psi, whereas the drying gas was set to a flow of 6 L min^{-1} at a temperature of 350°C. Mass spectra were obtained using the DataAnalysis program for LC/MSD Trap version 3.2 (Bruker Daltonik GmbH, Germany). For quantification of Z, ZR, ABA, and IAA, calibration curves were constructed for each analyzed component (0.05, 0.075, 0.1, 0.2, and 0.5 mg L^{-1}) and corrected for 0.1 mg L^{-1} internal standards: [$^2H_5$]*trans*-zeatin, [$^2H_5$]*trans*-zeatin riboside, [$^2H_6$]*cis*,*trans*-abscisic acid (Olchemin Ltd, Olomouc, Czech Republic), and [$^{13}C_6$]indole-3-acetic acid (Cambridge Isotope Laboratories Inc., Andover, MA, USA). Recovery percentages ranged between 92 and 95%.

Enzyme Extraction and Assay

Fresh leaf or root tissue samples (500 mg) were frozen with liquid nitrogen and stored at −20°C until analysis. A total of 500 mg of plant tissue was pulverized under liquid nitrogen with a chilled pestle and mortar and then homogenized with acid-washed sand and 5 ml ice-cold enzyme extraction buffer containing 50 mM HEPES–KOH, pH 7.5; 10 mM $MgCl_2$, 1 mM Na_2EDTA, 2,6 mM dithiothreitol (DTT), 10% ethylene glycol, 0.02% Triton X-100, 50 µM leupeptin, 0.5 mM phenylmethylsulfonyl (PMSF), and 1 g of PVPP (polyvinylpolypyrrolidone). After centrifugation at 15,000 *g* at 4°C, the supernatant was collected and used for enzyme assays. The activity of NADH-GOGAT (NADPH-dependent glutamine:2-oxoglutarate amidotransferase, EC 1.4.1.13) was assayed spectrophotometrically according to Groat and Vance (1981) by monitoring the oxidation of NADH at 340 nm. The activity of glutamine synthetase (GS, EC 6.3.1.2) was determined by transferase reaction, which measures the formation of γ-glutamyl hydroxymate at 540 nm, as described by Kaiser and Lewis (1984).

A modified in vivo NO_3^- reductase assay (Goupil and others 1998) was used to estimate the activity of NR (nitrate reductase, EC 1.6.6.1). Fresh tissue (100–200 mg) was placed into 3 ml of 50 mM KH_2PO_4 buffer (pH 7.5) and 100 mM KNO_3, containing 1.2% propanol. The assay was carried out in the presence of 100 mM KNO_3 in the medium. Incubation took place in the dark at 30°C, and a 0.5-ml subsample was taken from the medium 15 and 30 min after the start of the incubation to measure the NO_2^- production. The subsamples were ice cooled and 0.5 ml of sulfanilamide (1% w/v in 3 M HCl) was added. Five minutes after, 0.5 ml of N-(1-nil) ethylene diamine (0.02%) was also added and NO_2^- production was measured at 540 nm.

To measure cytokinin oxidase/dehydrogenase (CKX, EC 1.5.99.12) activity, leaf samples were cut into small

pieces, powdered with liquid nitrogen using a pestle and mortar, and extracted with a 1.5-fold excess (v/w) of 0.2 M Tris/HCl buffer (pH 8.0), containing 1 mM phenylmethanesulfonyl fluoride and 3% Triton X-100. Tissue debris was removed by centrifugation at 12,000 *g* for 10 min. The extract was loaded onto a Sephadex G-25 (50 × 2.5 cm) column equilibrated with 0.1 M Tris/HCl (pH 8.0) to remove the low-molecular-mass fraction. The protein fraction was then used to assay CKX activity. The assay was performed according to the method described by Frébort and others (2002), with some modifications. Samples were incubated in a reaction mixture (total volume = 0.6 ml in an Eppendorf tube) of 100 mM reaction buffer (imidazole/HCl buffer, pH 6.0), 0.5 mM electron acceptor [2,6-dichloroindophenol], and 0.5 mM substrate for 0.5–12 h at 37°C. All specific enzymatic activities were expressed as pkat mg^{-1} protein.

Statistical Analysis

Data were subjected to an analysis of variance (ANOVA II) that considered salinity and nitrogen forms as well as their interactions as main factors using the SAS software (SAS System for Windows, version 8.02), with mean discrimination achieved using the Student–Newman–Keuls test at the 5% level.

Results

Plant Growth and Biomass Allocation

In the absence of salt, the NO_3^-/NH_4^+ ratio in the hydroponic media did not significantly affect plant biomass (Fig. 1a, b). However, after 3 weeks under salt stress, both shoot and root fresh weights were reduced to a lesser extent under the 5/1.5 treatment than under the 6/0.5 treatment. Salinized plants growing under the ammonium-enriched treatment (5/1.5) produced 20% more biomass than under the normal Hoagland solution (6/0.5) by the end of the experiment. Because the positive effect of ammonium enrichment was more marked on the shoot than on the roots, the expected increase of the root/shoot ratio under salinity was lower (50%) in the 5/1.5 than in the 6/0.5 treatment (Fig. 1c).

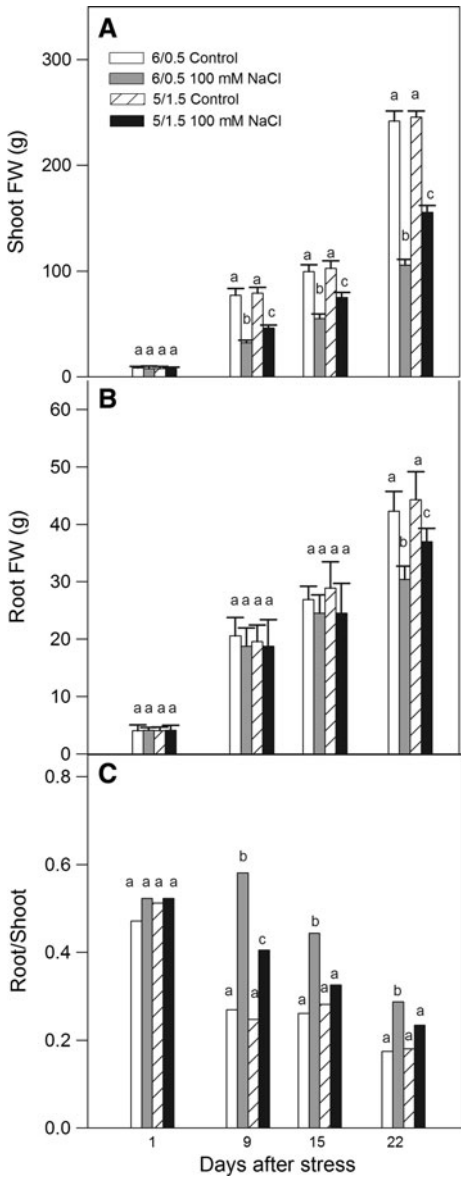

Fig. 1 Shoot (**a**), root (**b**) biomass and root/shoot ratio (**c**) in tomato plants (cv Moneymaker) grown for 3 weeks on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (*white* and *hatched bars*) or presence of 100 mM NaCl (*gray* and *black bars*). Data are means of 10 plants ± SE. *Different letters* indicate significant differences between treatments according to Student–Newman–Keuls test at $P < 0.05$

Chlorophyll Fluorescence and Stomatal Conductance

In the absence of salt, the NO_3^-/NH_4^+ treatment did not significantly change the maximum quantum efficiency of PSII (F_v/F_m) (Fig. 2a). However, under salt stress, the chlorophyll fluorescence parameter sharply decreased after 15 days of salinization under 6/0.5 treatment, although it was not significantly affected in the salinized plants growing under ammonium-enriched media (5/1.5) (Fig. 2a). From day 9 until the end of the experiment, salinity reduced leaf stomatal conductance but the form of N nutrition had no significant impact on this parameter (Fig. 2b).

Sodium and Potassium Concentration

Under salinity, Na^+ concentrations continuously increased with time in leaf 4 and roots until reaching similar

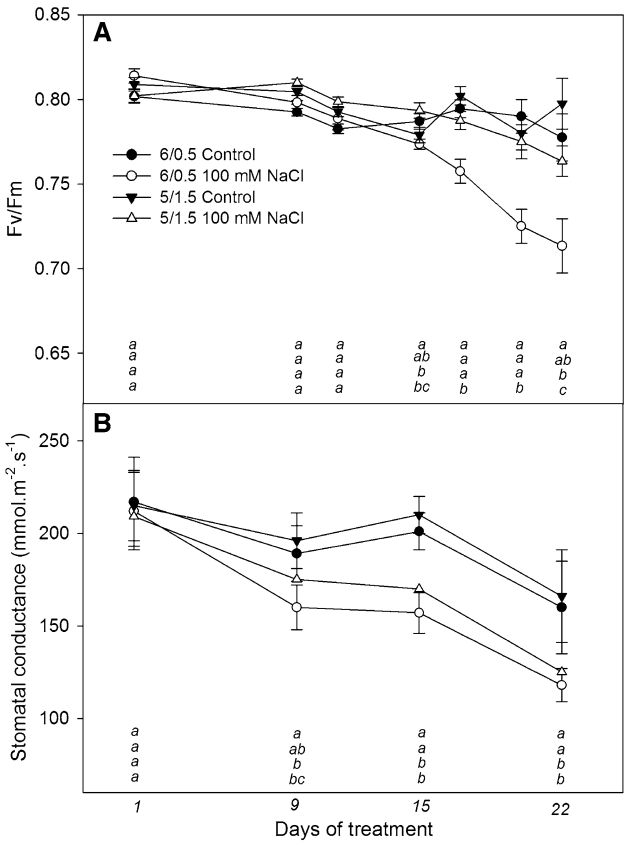

Fig. 2 Maximum photochemical efficiency (F_v/F_m) (**a**) and stomatal conductance (g_s) (**b**) in leaf 4 of tomato (cv Moneymaker) grown for 3 weeks on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (*closed circles* and *triangles*) or presence of 100 mM NaCl (*open circles* and *triangles*). Data are means of 6 plants ± SE. *Different letters* indicate significant differences between treatments for a given date according to Student–Newman–Keuls test at $P < 0.05$

final values under both N conditions, although the initial accumulation (first 10 days) was slower under ammonium-enriched (5/1.5) conditions (Fig. 3a, d). Potassium concentrations were strongly decreased (by up to 65% at the end of the experiment) with salinization time in the leaves and, to a lesser extent, in the roots. However, these ionic changes were attenuated under ammonium-enriched (5/1.5) conditions (Fig. 3b, e). Indeed, at the end of the salinization period, plants cultivated under the 5/1.5 treatment had a 30% higher K^+ concentration in leaf 4 than the plants grown in the Hoagland medium (6/0.5).

The K^+/Na^+ ratio decreased with age in control leaves and roots (Fig. 3c, f) and was strongly decreased already after 24 h of salinization. The impact of nitrogen form on this parameter was limited to short-term exposure because K^+/Na^+ ratios remained slightly higher under enriched ammonium (5/1.5) than in normal nutrient solution at the beginning of salt treatment only.

Nitrate and Ammonium Concentrations

Plant N status was determined by analyzing the nitrate and ammonium concentrations in leaf 4 and roots at the end of the experiment (Table 1). Under control conditions, ammonium enrichment (5/1.5) decreased NO_3^- concentration only in the roots (18%) but had no effect on leaf 4. Salinity decreased NO_3^- concentration in both leaf 4 and roots by 30 and 50% in the standard Hoagland's treatment (6/0.5), whereas in the ammonium-enriched treatment (5/1.5), salinity reduced leaf NO_3^- concentration but increased (2.5-fold) root NO_3^- concentration. In salt-treated plants, leaf NO_3^- was clearly higher in the 5/1.5 than in the 6/0.5 treatment.

Under control conditions, ammonium enrichment (5/1.5) significantly increased leaf NH_4^+ concentrations but had no effect on root NH_4^+ concentrations. Salinity strongly increased NH_4^+ concentration in roots and leaves by 50–60% regardless of the nitrogen treatment. As a consequence, the NH_4^+ concentrations in both roots and leaves of the salinized plants were 25 and 50% higher in the 5/1.5 treatment than in the 6/0.5 one, respectively.

Enzymes of Nitrogen Metabolism

Nitrate reductase (NR) activity in the leaves was higher than in the roots regardless of both nitrogen and salt treatments (Table 2). Ammonium enrichment (5/1.5) increased NR activities in the roots but strongly decreased it in the leaves. Salinity significantly increased root NR activity (50%) but decreased it in the leaves (60%) in the standard Hoagland's solution (6/0.5), whereas in the ammonium-enriched (5/1.5) solution, salinity had no effect on root NR activity and decreased leaf NR activity to a lesser extent (40%) (Table 2).

Under control conditions, there was no significant difference in leaf GOGAT activity between the two nitrogen treatments (Table 2), but ammonium enrichment increased root GOGAT activity 30%. Salinity decreased leaf GOGAT activity under Hoagland's N conditions (6/0.5) but not under ammonium-enriched conditions, whereas an opposite trend was recorded for root GOGAT activity.

Under control conditions, ammonium enrichment (5/1.5) increased leaf GS activity by twofold compared to the standard Hoagland's solution (6/0.5) (Table 2). Salinity induced an additional 40% increase in leaf GS activity compared to unstressed controls regardless of the N treatment. No GS activity was detected in the roots (Table 2).

Hormonal Profiling and CKX Activity

It is noteworthy that nitrogen form had no impact on ABA (Fig. 4), IAA (Fig. 5), or CK (Fig. 6) concentrations in any plant compartment in the absence of salt stress.

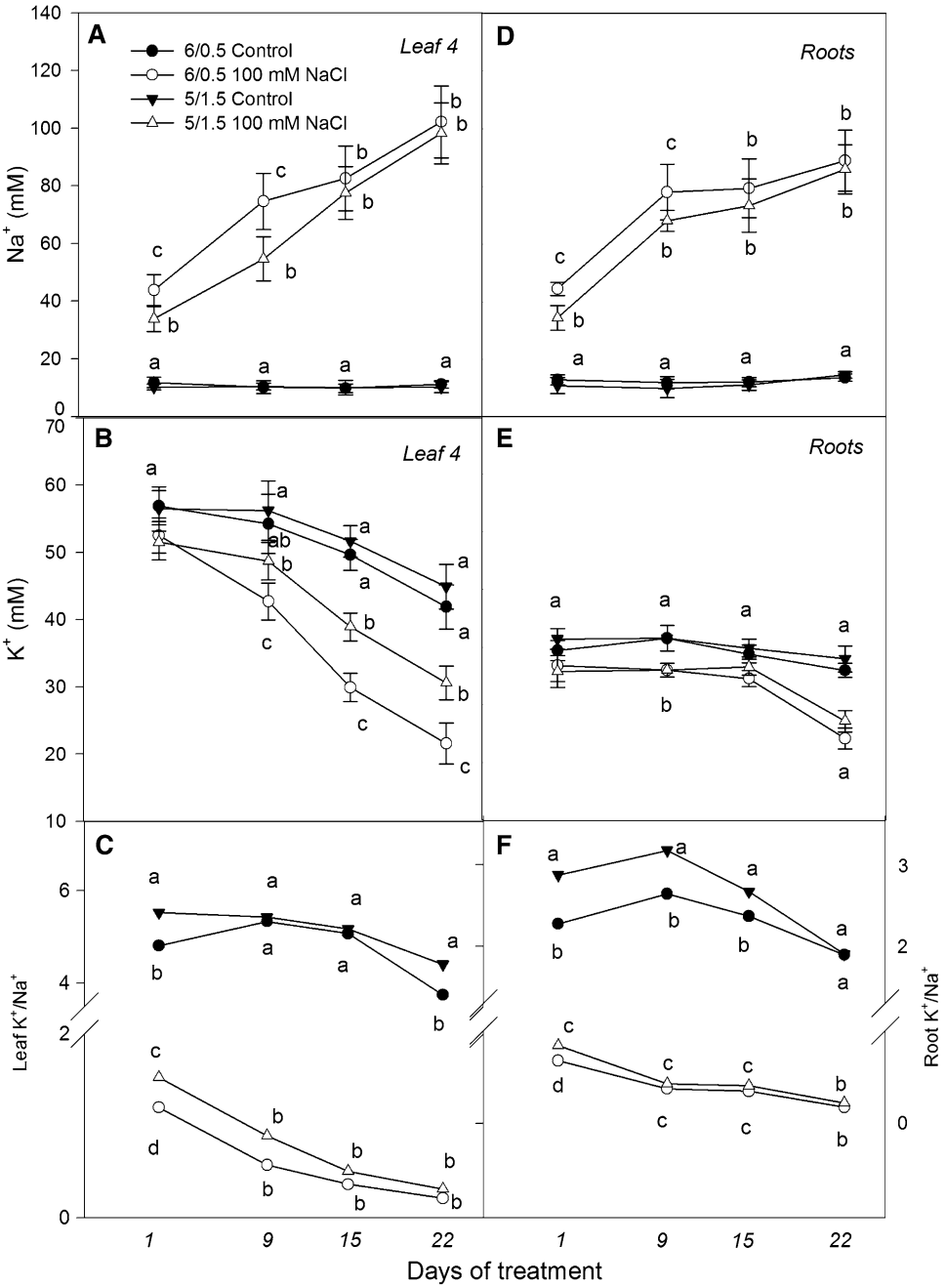

Fig. 3 Sodium (**a**, **d**) and potassium (**b**, **e**) concentrations, and potassium/sodium ratio (**c**, **f**) in leaf 4 (**a**–**c**) and roots (**d**–**f**) of tomato plants (cv Moneymaker) grown for 3 weeks on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (*closed circles* and *triangles*) or presence (*open circles* and *triangles*) of 100 mM NaCl. Data are means of 6 plants ± SE, with *different letters* above and below the symbols as in Fig. 2

Table 1 NO_3^- and NH_4^+ concentrations of leaf 4 and roots of tomato plants (cv Moneymaker) grown for 3 weeks on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (control) or presence of 100 mm NaCl (salt)

		6/0.5		5/1.5	
		Control	Salt	Control	Salt
NO_3^- (mM)	Roots	$16.81^a \pm 1.89$	$8.13^b \pm 0.99$	$13.56^c \pm 2.10$	$19.95^d \pm 0.60$
	Leaf 4	$28.21^a \pm 1.6$	$19.12^b \pm 2.12$	$27.76^a \pm 1.02$	$23.00^c \pm 2.09$
NH_4^+ (mM)	Root	$0.61^a \pm 0.22$	$1.20^b \pm 0.12$	$0.76^a \pm 0.10$	$1.50^c \pm 0.18$
	Leaf 4	$0.75^a \pm 0.12$	$1.35^b \pm 0.01$	$1.12^c \pm 0.01$	$2.84^d \pm 0.40$

Data are means of 10 plants ± SE. Different *superscript letters* indicate significant differences between treatments according to Student–Newman–Keuls test at $P < 0.05$

Table 2 Enzymatic activities for nitrate reductase (NR), NADH-GOGAT (NADPH-dependent glutamine:2-oxoglutarate amidotransferase), and glutamine synthetase (GS) in roots and leaf 4 in tomato plants (cv Moneymaker) grown for 3 weeks on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (control) or presence of 100 mM NaCl (salt)

		6/0.5		5/1.5	
		Control	Salt	Control	Salt
NR (nkat g^{-1} FW)	Roots	$0.0045^{a} \pm 0.0001$	$0.0079^{b} \pm 0.0001$	$0.0069^{c} \pm 0.0001$	$0.0063^{c} \pm 0.0001$
	Leaf 4	$0.12^{a} \pm 0.02$	$0.05^{b} \pm 0.01$	$0.05^{b} \pm 0.01$	$0.03^{c} \pm 0.01$
GOGAT (pkat mg^{-1} protein)	Root	$0.32^{a} \pm 0.04$	$0.29^{a} \pm 0.10$	$0.46^{c} \pm 0.07$	$0.39^{d} \pm 0.02$
	Leaf 4	$0.17^{a} \pm 0.13$	$0.13^{b} \pm 0.02$	$0.19^{a} \pm 0.02$	$0.18^{a} \pm 0.04$
GS (pkat mg^{-1} protein)	Root	ND	ND	ND	ND
	Leaf 4	$3.56^{a} \pm 0.80$	$5.3^{b} \pm 0.26$	$7.23^{c} \pm 1.30$	$10.2^{d} \pm 0.43$

Data are means of 10 plants ± SE. Different *superscript letters* indicate significant differences between treatments according to Student–Newman–Keuls test at $P < 0.05$. ND = not detectable

In the presence of NaCl, ABA concentrations in roots, xylem sap, and leaf 4 increased linearly with duration of the treatment but to a lesser extent in the enriched ammonium N treatment (Fig. 4).

Salinity induced opposite changes in IAA concentrations in leaves and roots but to a different extent depending on the N treatment. Salinity decreased leaf IAA concentrations between 50% (day 9) and 90% (day 22) under Hoagland's N nutrition (6/0.5), whereas the decrease was less marked (57% at day 22) under enriched ammonium nutrition (Fig. 5a). In the roots, salinity increased the auxin concentration by 1.5- to 2-fold compared to unsalinized plants but to a greater extent in the 5/1.5 treatment (Fig. 5b).

Salinity decreased leaf and xylem sap *t*Z concentrations but increased root *t*Z concentrations irrespective of the nitrogen treatment (Fig. 6a–c). Salinity also decreased *t*ZR concentrations in all plant compartments (Fig. 6d–f) but to a lesser extent under ammonium enrichment (5/1.5) than under the standard Hoagland's treatment (6/0.5). However, only a marginal impact of N form on leaf *t*Z concentration was detected in the leaf after prolonged salinization (Fig. 6a).

No significant effect of N treatments was recorded for CKX activity (Fig. 7) in plants maintained in the absence of salt. Salinity increased leaf CKX activity only by day 22, and it was twofold higher under the 6/0.5 than under the 5/1.5 N treatment.

Discussion

Increasing the Nutrient Solution NH_4^+ Proportion Increases Tomato Vegetative Growth Under Saline Conditions

Under control (nonsaline) conditions, the NO_3^-/NH_4^+ ratio had no significant effect on shoot and root fresh weight (Fig. 1a, b). Under saline conditions, however, decreasing the NO_3^-/NH_4^+ ratio from 6/0.5 to 5/1.5 significantly increased biomass production by 22% (Fig. 1a, b), thus confirming previous observations on salt-stressed tomato (Flores and others 2001) and barley (Kant and others 2007). Stimulatory effects on plant growth by low concentrations of NH_4^+ have been associated with energy savings (Raven 1985) because NO_3^- must be reduced to NH_4^+ (an energy-dependent process requiring 20 molecules of ATP) before incorporation into amino acids. In contrast, NH_4^+ assimilation requires only five molecules of ATP (Raven 1985). Energy saving during N assimilation when the 5/1.5 NO_3^-/NH_4^+ ratio was used might allow more resources to be allocated to energy-requiring salt stress tolerance mechanisms (Kant and others 2007). Although PSII efficiency (F_v/F_m) decreased after 15 days of salt treatment with the normally used Hoagland solution (6/0.5 NO_3^-/NH_4^+) (Fig. 2), F_v/F_m did not show any significant differences between control and salt-stressed plants growing under the 5/1.5 NO_3^-/NH_4^+ treatment after 22 days of salt treatment. This indicates that salt-induced senescence was delayed by increasing NH_4^+ fertilization from 0.5 to 1.5 mM.

These beneficial impacts were related to decreased Na^+ accumulation only at the beginning (<15 days) of salt stress (Fig. 3a, d). Competition between NH_4^+ and Na^+ for root uptake sites has been suggested as the cause of the decrease in Na^+ uptake and transport from roots to shoots in ryegrass (Sagi and others 1998) and in barley (Kant and others 2007). Growth stimulation induced by the 5/1.5 NO_3^-/NH_4^+ treatment after 9 days (Fig. 1a) could dilute Na^+ accumulation, thus transiently lowering Na^+ concentration. However, because salinity increased Na^+ concentrations by 80–90%, the slight reduction in leaf Na^+ concentration by ammonium enrichment is unlikely to be solely responsible for mitigation of salt stress effects. More interestingly, increasing the NH_4^+ proportion from 0.5 to 1.5 also clearly reduced the salt-induced decrease in K^+ concentrations in leaves (Fig. 3b, d). Nitrate transport to

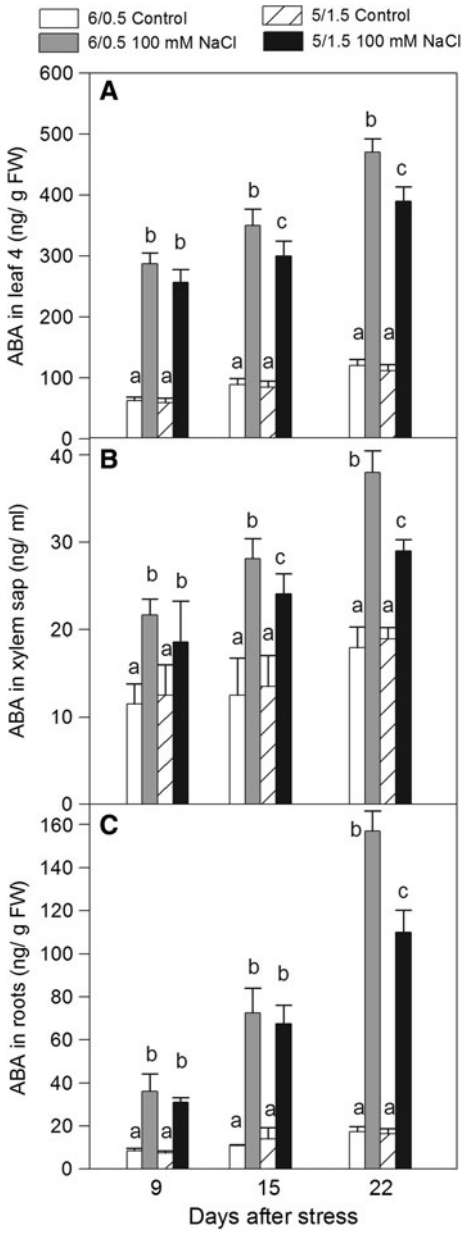

Fig. 4 Abscisic acid (ABA) concentration of leaf 4 (**a**), xylem sap (**b**), and roots (**c**) of tomato plants (cv Moneymaker) grown for 3 weeks on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (*white* and *hatched bars*) or presence of 100 mM NaCl (*gray* and *black bars*). Data are means of 10 plants ± SE, with *different letters* above the bars as in Fig. 1

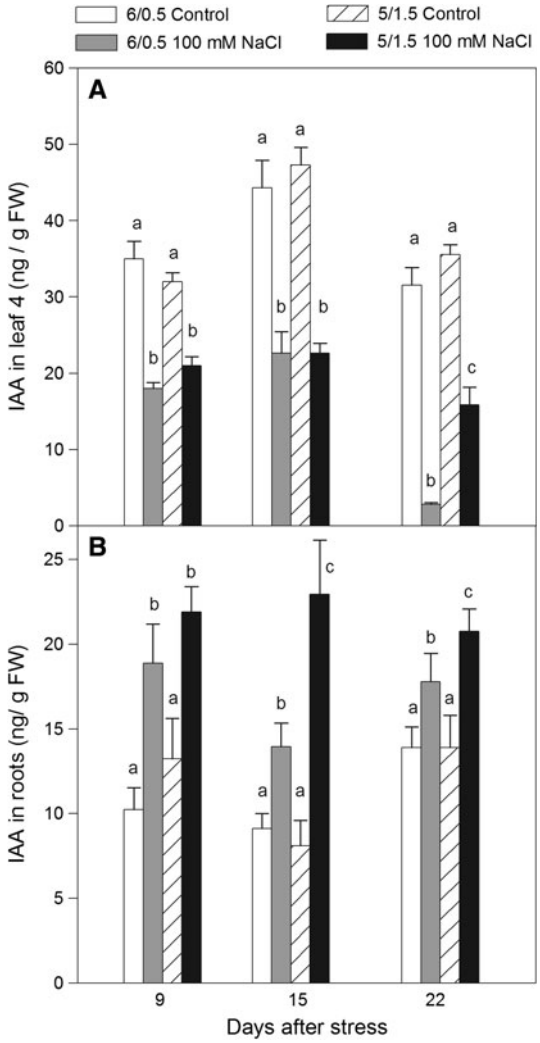

Fig. 5 IAA concentration of leaf 4 (**a**) and roots (**b**) of tomato plants (cv Moneymaker) grown for 3 weeks on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (*white* and *hatched bars*) or presence of 100 mM NaCl (*gray* and *black bars*). Data are means of 10 plants ± SE, with *different letters* above the bars as in Fig. 1

the shoot depends on K^+ shuttle operation, which coordinates nitrate uptake and nitrate utilization because K^+ acts as a counterion for the transport of NO_3^- to the leaves via xylem, and of malate to the root via phloem to sustain N assimilation and other assimilate-dependent processes. Salt stress or drought usually blocks K^+ shuttle activity, inhibiting the transport of nitrate to the shoot as a consequence of a stress-induced decrease in K^+ (Cruz and others 1993). Hence, the improved K^+ status under saline conditions in NH_4^+-enriched solution could, at least partly, explain the higher NO_3^- concentration in leaves of salt-treated plants in the 5/1.5 compared to the 6/0.5 treatment (Table 1) as well as the increase in the assimilatory activity of GOGAT in the roots (Table 2). The improvement in K^+ nutrition may be related to the improved CK status, as discussed below.

Increasing the Nutrient Solution NH_4^+ Proportion Improves Nitrogen Assimilation and/or Reassimilation Pathways Under Saline Conditions

Despite the energy cost, plants have adapted to NO_3^- as the most common form of available N in agricultural soils.

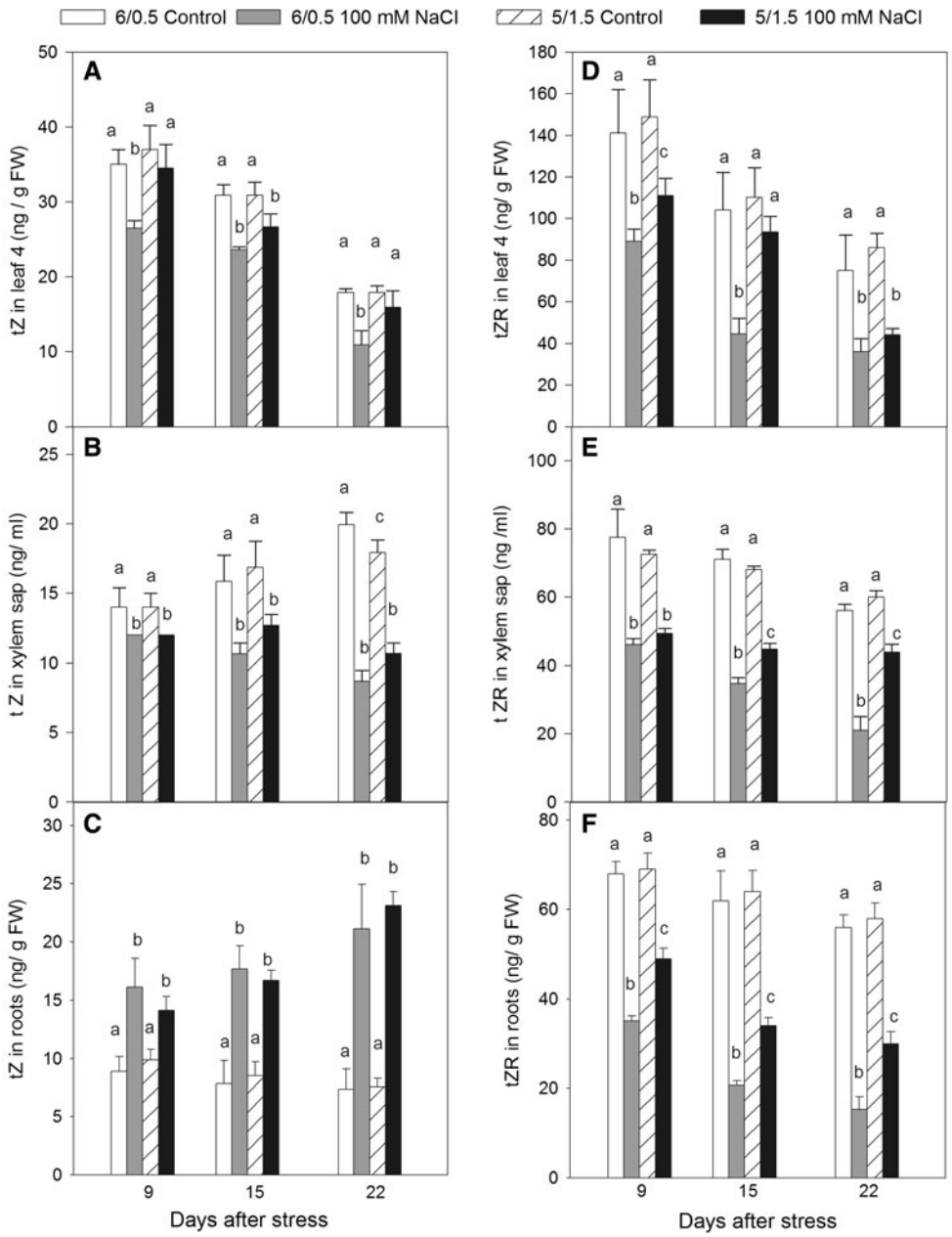

Fig. 6 Cytokinin (Z, ZR) concentrations in leaf 4 (**a**, **d**), xylem sap (**b**, **e**), and roots (**c**, **f**) of tomato plants (cv Moneymaker) grown for 3 weeks on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (*white* and *hatched bars*) or presence of 100 mM NaCl (*gray* and *black bars*). Data are means of 5 plants ± SE, with *different letters* above the bars as in Fig. 1

One reason is that when NH_4^+ is provided at relatively high concentrations (>10 mM), its uptake may exceed the assimilatory capacity or change the ionic equilibria leading to toxicity (Flores and others 2001; Lu and others 2009). Such effects were not evident here as the maximum NH_4^+ concentration was only 1.5 mM. Salt-treated plants growing under the 5/1.5 NO_3^-/NH_4^+ treatment had higher levels of both NO_3^- and NH_4^+ compared to plants growing on the 6/0.5 NO_3^-/NH_4^+ treatment (Table 1); this suggests a better ability to absorb and transport N in general.

Salt stress enhances reduction of nitrate (NR activity) in the root (Table 2). The resulting ammonium could be assimilated and amino acids (especially gln and asn) transported to the shoot through the xylem (Pérez-Alfocea and others 2000). Under standard 6/0.5 NO_3^-/NH_4^+ solution, salinity therefore shifts the main location of nitrate assimilation from the shoot to the root, which induces a higher demand for carbon skeletons in the roots and a corresponding reduction in growth rate. In contrast, under enriched NH_4^+ solution, salinity reduced NR activities in both leaves and roots, and exogenous NH_4^+ could thus supply NH_4^+ requirements, as suggested by a clear NaCl-induced increase in NH_4^+ concentration in both roots and leaves (Table 1).

Increasing the proportion of NH_4^+ in the nutrient solution also increased GS activity in the leaf in both nonsaline

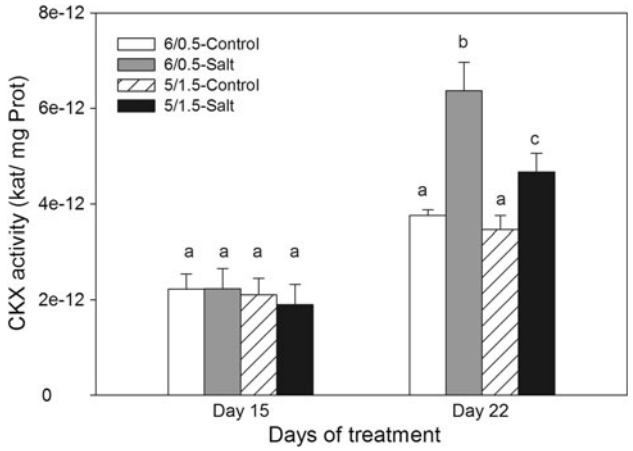

Fig. 7 Cytokinin oxidase/dehydrogenase (CKX) activities (pkatal mg^{-1} protein) in leaf 4 of tomato plants grown for 15 and 22 days on two different nitrate/ammonium ratios (6/0.5 or 5/1.5) in half-strength Hoagland medium in the absence (control) or presence of 100 mM NaCl (salt). Data are means of 5 plants ± SE, with *different letters* above the bars as in Fig. 1

and saline conditions compared to normal Hoagland solution (Table 2). GS activity increased in response to saline conditions in numerous plant species, including tomato (Misra and Dwivedi 1990; Cramer and others 1995). GS activity increased in five different species under nonsaline conditions when NH_4^+ instead of NO_3^- was used as a nitrogen source (Arnozis and others 1988). In contrast, nitrogen ionic form had no effect on ryegrass GS activity in both nonsaline and saline conditions (Sagi and others 1998). An increased GS activity may also facilitate the production of glutamine, proline, and other organic solutes, characteristic of osmotic and pH adjustments under stress conditions (Lorenzo and others 2001).

In contrast, GOGAT activity decreased under salt stress conditions but in different organs, depending on the ammonium treatment. Absolute GOGAT and GS activities of salt-treated plants were significantly higher for plants grown on NH_4^+-enriched nutrient solution than on normal 6/0.5 NO_3^-/NH_4^+. This better ability of N assimilation could be related to a better recycling of carbon skeletons under the effect of diverse aminotransferases and GABA cycle, which generate CO_2 (nonphotosynthetic fixation through PEP carboxylase) and other intermediates of the Krebs's cycle like succinate (Temple and others 1998). Globally, increased ammonium nutrition increased both N assimilation in roots (GOGAT) and the reassimilatory pathway in leaves (GS). These results suggest a higher efficiency of the ammonium-producing photorespiratory pathway, which could help to protect leaves from stress-induced photoinhibition and photooxidation, as supported by the maintenance of PSII efficiency (Fig. 2a).

Increasing NH_4^+ Fertilization Modified Ionic and Hormonal Status in Salt-treated Tomatoes

Additional NH_4^+ in the nutrient solution modified both root and shoot hormonal status in tomato but only in the presence of NaCl. Accordingly, for all measured hormone-related parameters, a significant positive interaction existed between salt and nitrogen form.

Ammonium enrichment of the nutrient solution decreased root, xylem, and leaf ABA concentration (Fig. 4) but increased root and leaf IAA concentration (Fig. 5), leaf *t*Z concentration (Fig. 6a), and xylem sap *t*ZR concentration (Fig. 6d) compared with normal 6/0.5 NO_3^-/NH_4^+. Most of these changes appeared 10 days after salinization and were not attributable to differences in leaf or root Na^+ concentration because ammonium enrichment reduced Na^+ accumulation at the beginning of stress exposure only (Fig. 3a, c). However, consistent differences in K^+ concentration between the two salinized treatments provide more compelling evidence that K^+ status may have been a primary regulator of hormone status or vice versa. Indeed, a higher K^+ concentration in NaCl-treated plants in the 5/1.5 NO_3^-/NH_4^+ treatment (Fig. 3) was correlated with higher concentrations of *t*Z and *t*ZR (Fig. 6). Similarly, rootstocks promoting vegetative vigor and delaying leaf senescence of salinized tomato had higher xylem K^+ and *t*ZR concentrations (Albacete and others 2009), suggesting a putative CK–K^+ interaction regulating plant salt stress responses.

Abscisic acid promotes both stomatal closure and sugar accumulation by blocking sucrose export from mature leaves, thus contributing to osmotic adjustment (Pérez-Alfocea and Larher 1995; Balibrea and others 2000; Dodd 2003). Although ABA concentrations continuously increased with duration of salinization, its accumulation was significantly lower in leaves of salt-stressed plants growing under 5/1.5 NO_3^-/NH_4^+ treatment compared to the normal Hoagland solution from day 15 (Fig. 4). However, this attenuation of ABA accumulation did not significantly increase stomatal conductance (Fig. 2) or transpiration rate, thus it did not attenuate xylem Na^+ delivery to the shoot and leaf Na^+ accumulation. The similar stomatal responses of the two salinized treatments, in spite of differences in ABA concentration (Fig. 4) and other hormones such as IAA and CKs thought to promote stomatal opening (Dodd 2003), also argues against a role for changes in these leaf hormone concentrations in directly regulating stomatal behavior.

Although hormonal interactions seemingly had no effect on stomatal conductance, it is plausible that the concentration of one hormone impacted the concentration of another. It has been suggested that ABA modifies CK concentrations and CKX activity and gene expression (Brugière and others 2003; Kudoyarova and others 2007). Furthermore, in wheat seedlings from which nutrients were

withheld, activation of CKX activity (and consequent decrease of cytokinin status) was attenuated by the simultaneous addition of fluridone (an ABA biosynthesis inhibitor) to the nutrient solution, suggesting ABA mediation of CKX activity (Vysotskaya and others 2009). Similarly, decreased ABA accumulation in the 5/1.5 NO_3^-/NH_4^+ treatment (Fig. 4a) was accompanied by decreased leaf CKX activity after 22 days (Fig. 7), which may be partially responsible for the increased leaf *t*Z concentration (Fig. 6a). Thus, the salt-induced ABA increase in leaves could be partially responsible for the decreased CK contents, thus indirectly enhancing the salt-induced senescence under normal Hoagland solution.

Nitrate can also directly induce root CK synthesis and CK translocation to the shoots via the xylem (Takei and others 2001; Sakakibara and others 2006), but the contribution of xylem CK delivery to shoot and leaf CK status in plants grown at different N status remains controversial (Dodd and others 2004; Dodd and Beveridge 2006). Ammonium enrichment increased both leaf *t*Z and *t*ZR concentrations of salinized plants at the beginning of stress exposure, and *t*Z concentrations were still higher in leaf 4 and xylem sap at the end of the treatment in plants exposed to 5/1.5 NO_3^-/NH_4^+ than in those exposed to 6/0.5 NO_3^-/NH_4^+ (Fig. 6a, d). Paradoxically, this result is consistent with nitrate-induced CK synthesis as both root NO_3^- concentration (Table 1) and root *t*ZR concentration (Fig. 6f) were higher in salinized plants under the 5/1.5 NO_3^-/NH_4^+ treatment. However, shoot processes may also be responsible for effects of ammonium enrichment on leaf CK concentrations, because leaf CKX activity was 27.5% higher in salinized plants grown in normal Hoagland solution than in NH_4^+-enriched solution. CKX activity may fine tune an optimal level of CKs for growth adaptation to the stress conditions (for example, shift in shoot to root assimilate partitioning or adjust growth potential to N availability) and/or reset a cytokinin signaling system to a basal level (Werner and others 2001). Irrespective of the mechanisms by which ammonium enrichment increased plant CK status in salinized plants, these increases may have delayed the onset of leaf senescence as cytokinin involvement in preventing leaf senescence has been demonstrated in different plant species (Balibrea and others 2004; Rivero and others 2007).

Ammonium enrichment also limited salt-induced decreases in leaf IAA concentration and increases in root IAA concentration (Fig. 5). Ammonium nutrition is known to induce aldehyde oxidase, which plays an important role in the synthesis of IAA (Koshiba and others 1996). As stated above, Gerendas and others (1997) postulated a link between increased auxin transport to the roots and increased root cytokinin production under ammonium nutrition. In contrast, ammonium feeding, in at least one case, suppressed root auxin content (Kudoyarova and others 1997). Increased root auxin accumulation under the 5/1.5 NO_3^-/NH_4^+ treatment may promote cell elongation and root growth and may be important in increasing root/shoot ratio under salinity (Havlová and others 2008). Moreover, auxin concentrations in leaves were significantly higher in salt-stressed plants growing under the 5/1.5 NO_3^-/NH_4^+ treatment compared to the normal Hoagland solution (Fig. 4) and this could also be related to the observed delay in leaf senescence because auxin has been generally seen as a senescence-retarding factor (Schippers and others 2007).

Conclusion

Interactions between nitrogen and cytokinin in regulating metabolism and development are usually considered in the absence of salt stress (Sakakibara and others 2006; Lu and others 2009), yet this work shows that salinity may influence those interactions. This work demonstrates (1) that increasing nutrient solution NH_4^+ concentration (5/1.5 NO_3^-/NH_4^+ treatment) improved plant growth and delayed senescence of salt-treated tomato compared to the usual low NH_4^+ concentration (Hoagland 6/0.5 NO_3^-/NH_4^+ treatment), and (2) that this improvement is associated with modification in ionic status (increased K^+/Na^+ ratio), N (re)assimilatory metabolism, and plant hormone status (namely, higher CK concentrations and lower ABA concentrations). More direct evidence of the involvement of changes in plant hormone status in mediating plant physiological responses to concurrent ammonium enrichment and salinity could be sought by exogenous application of ABA or cytokinins, their biosynthesis inhibitors, or inhibitors of ABA/CK-degrading enzymes such as ABA 8′-hydroxylase and CKX. In addition, both the hormone and N status of mutants/transgenics in either N metabolism or hormone biosynthesis/catabolism pathways should be investigated in response to salinization.

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