

## SALINITY STRESS

# Evapotranspiration as a Criterion to Estimate Nitrogen Requirement of Maize Under Salt Stress

C. F. Lacerda<sup>1</sup>, J. F. S. Ferreira<sup>2</sup>, X. Liu<sup>2</sup> & D. L. Suarez<sup>2</sup><sup>1</sup> Department of Agricultural Engineering/Universidade Federal do Ceará and INCTSal/CNPq, Fortaleza, Ceará, Brazil<sup>2</sup> USDA-ARS U.S. Salinity Laboratory, Riverside, CA, USA**Keywords**evapotranspiration; N loss; nutrient demand; photosynthesis; salinity; *Zea mays* L.**Correspondence**

C. F. Lacerda  
Department of Agricultural Engineering/  
Universidade Federal do Ceará and  
INCTSal/CNPq  
Campus do Pici  
Bloco 804, 60.455-760  
Fortaleza  
Ceará  
Brazil  
Tel.: +55 85 3366 9127  
Fax: +55 85 3366 9754  
Email: cfeitosa@ufc.br

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**Abstract**

We tested the hypothesis that by reducing the application of N, based on the decrease in evapotranspiration (ET) expected due to increase in soil salinity, it is possible to reduce N loss without causing N deficiency or further yield loss in salt-stressed maize plants. We tested four levels of salinity of irrigation water (S1 = 0.5; S2 = 2.5; S3 = 5.0; and S4 = 7.5 dS m<sup>-1</sup>) and four N rates using outdoor soil columns with five replicates. The N rates were as follows: N1: N recommendation for maize (2.6 g per column); N2: 0.3 times the N recommendation (0.78 g per column); N3: reduction in N1 based on the decrease in ET caused by salinity; and N4: reduction in N2 based on the decrease in ET caused by salinity. The amounts of N for N3 and N4 were reduced (in relation to N1 and N2) by 7 %, 15 % and 30 % for 2.5, 5.0 and 7.5 dS m<sup>-1</sup>, respectively. Salinity caused NO<sub>3</sub><sup>-</sup> accumulation in the soil, plant growth inhibition and stomatal closure. The low rates of N (N2 and N4) did not meet the N demand of maize plants, especially for low levels of salinity (control and 2.5 dS m<sup>-1</sup>). On the other hand, based on the available growth data, physiological responses and nutritional status, one can conclude that plants under N1 and N3 had the same potential for final yield. For these N rates, reduction in N application according to ET (N3 rate) not only allowed plant growth and maize physiological responses, but also increased N-use efficiency and greatly reduced soil nitrate accumulation compared to N1 rate, at the same levels of salinity. We conclude that reduction in N application, based on reductions in ET, is a good strategy to reduce both the risk of ground water contamination by NO<sub>3</sub><sup>-</sup> leaching and fertilization costs, without causing additional damage to plant development under salt stress.

**Introduction**

The interaction between salinity and mineral nutrition is a complex process because it involves more than 20 chemical elements, which in turn present different responses according to the interaction between environmental conditions and plant species. Despite this complexity, some authors report that salt tolerance can be increased by increasing the levels of certain nutrients, such as N, K and P. However, inhibition of plant growth by salinity is mainly due to osmotic and toxic effects imposed by the presence of salts in the root environment (Munns 2002, Lacerda et al. 2003). Although positive response to supplemental nutrient can be observed in salt-stressed plants, especially in low soil fertility conditions (Grattan and Grieve 1999),

this response is not higher than that recorded for plants under optimal, or non-saline conditions (Irshad et al. 2008, Semiz et al. 2014), resulting in nutrient loss to the environment instead of plant growth and development.

The inhibition of plant growth and damage to physiological processes caused by salinity reduce the amount of nutrients extracted from the soil (Shenker et al. 2003, Neves et al. 2009, Segal et al. 2010, Ramos et al. 2012). As a consequence, a large part of added nutrients are lost by leaching, causing contamination of the water table (Shenker et al. 2003, Segal et al. 2010, Ramos et al. 2011, 2012). Of course, ground water contamination will depend on the soil mobility and concentration of the nutrient, leaching fraction, depth of water table and preferential water flow, which can accelerate the migration rate of

nutrients causing them to bypass the soil matrix (Segal et al. 2010). These problems can be exacerbated for nutrients with high mobility in the soil, such as nitrate.

The existing recommendation regarding the addition of mineral nutrients to meet plant demand is based on crops maintained under ideal agronomic conditions, but these data do not reflect the actual nutrient demand of plants growing under adverse field conditions. For example, in extensive areas around the world, plants are submitted to salt or water stress and, consequently, are not able to use applied nutrients efficiently, even if these minerals are provided in excess of plant needs to reach full potential yield.

Although several researchers demonstrated that salinity reduced the total amount of nutrient extracted from the soil by crops (Shenker et al. 2003, Neves et al. 2009, Segal et al. 2010, Ramos et al. 2011, 2012, Zhang et al. 2012), no practical solution has been found either to improve nutrient absorption under salinity stress or to determine the real nutrient requirement of the crop under salt stress. One possible strategy to be tested is the application of nutrients in proportion to the amount of water consumed by crops, that is based on their evapotranspiration (ET) in response to salinity. Salinity reduces plant ET, and there is a good relationship between water consumption and plant yield. In the case of N, for example, one can expect a positive correlation between ET and the amount of N extracted from the soil, considering that the absorption of this nutrient is mainly due to mass flow process. So, positive correlation between ET and nitrogen accumulation in plants has been documented (Shenker et al. 2003, Feng et al. 2005, Ramos et al. 2012, Wang et al. 2012). In addition, reduction in  $\text{NO}_3^-$  uptake appears to be more associated with reduced water use than with chloride antagonism from salt stress (Lea-Cox and Syvertsen 1993, Abdelgadir et al. 2005).

Plants under optimal soil conditions reach their maximum ET, and their total water use depends on intrinsic characteristics of plant species, growth stage and local climate. Under water and salt stress, ET decreases to a value that is related to the extent of water shortage caused by these stressful factors (Pereira et al. 2007). Salinity decreases water absorption as a consequence of osmotic stress and, under this condition, the use of stress coefficient to reduce water application in irrigation was tested (Pereira et al. 2007), aiming to increase the water-use efficiency (WUE) by salt- and water-stressed plants.

Considering that salinity reduces both the consumption of water and the demand of nutrients by plants, our hypothesis is that reducing the supply of N, based on the decrease in ET expected by increase in soil salinity, it is possible to reduce N loss and to promote N-use efficiency (NUE) without causing N deficiency in salt-stressed maize plants. The goal of this work was to test the use of ET as a

criterion to estimate nitrogen requirement by salt-stressed maize plants.

## Material and Methods

### Experimental conditions

The experiment was conducted at the US Salinity Laboratory (ARS – USDA), Riverside, CA (33°59'N; 117°21'W), from September 13 to November 26. During the experiment, the mean maximum, minimum and average air temperature were 26.7, 12.8 and 20.0 °C, respectively. Maize plants were grown in columns of polyvinyl chloride (PVC) with 20 cm in diameter and 100 cm in length. Columns were filled by a sieved (5-mm mesh) sandy loam soil, pH 6.8 and non-saline soil ( $\text{EC}_e$  of 1.6  $\text{dS m}^{-1}$ ), collected near the experimental area. A nylon mesh and a cap adapted with a drainage pipe were used at the bottom of each PVC tube to retain the soil, but allowing the drainage water to be collected into 1-l glass bottles with wide mouths (Zhou et al. 2006) set below the drainage pipes.

### Experimental design and treatments

The experiment was conducted in a complete randomized block design following a  $4 \times 4$  factorial arrangement, composed of four levels of salinity ( $S_1 = 0.5$ ;  $S_2 = 2.5$ ;  $S_3 = 5.0$ ; and  $S_4 = 7.5 \text{ dS m}^{-1}$ ) and four N rates, with five replications. Low salinity water (control) was obtained from a well located in the experimental area having electrical conductivity ( $\text{EC}_w$ ) of 0.5  $\text{dS m}^{-1}$ . Treatments with salinity levels higher than the control were prepared by adding NaCl,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  salts in a 7 : 2 : 1 equivalent ratio, according to the relationship between  $\text{EC}_w$  and concentration ( $\text{mmol}_c \text{ l}^{-1} = \text{EC}_w \times 10$ ). Evapotranspiration measurements were based on the principle of the water balance in the soil column, that is ET was calculated by difference between water applied and water drained (leachate) during successive events of irrigation, performed every other day. During the experiment, two rain events were observed (13 and 25 mm). At the end of the experiment, the average leaching fraction for salinity treatments  $S_1$ ,  $S_2$ ,  $S_3$  and  $S_4$  were, respectively, 0.16, 0.17, 0.19 and 0.23, considering both irrigation and rainfall events.

The four N rates, applied as urea and potassium nitrate, were as follows: N1: N recommendation for maize in California ( $206 \text{ kg ha}^{-1}$ ); N2: 0.3 times the N recommendation for maize in California ( $62 \text{ kg ha}^{-1}$ ); N3: reduction in N1 based on the decrease in ET caused by salinity in the previous stage; and N4: reduction in N2 based on the decrease in ET caused by salinity in the previous stage. The total N applied for different treatments are presented in Table 1.

**Table 1** Rates of nitrogen (g per column) applied for different salt treatments

ECw (dS m <sup>-1</sup> )	Rates of nitrogen <sup>1</sup>							
	N1		N2		N3		N4	
	N-KNO <sub>3</sub> (Total N)	N-Urea						
0.5	0.45 (2.60)	2.15	0.45 (0.78)	0.33	0.45 (2.60)	2.15	0.45 (0.78)	0.33
2.5	0.45 (2.60)	2.15	0.45 (0.78)	0.33	0.45 (2.42)	1.97	0.45 (0.725)	0.275
5.0	0.45 (2.60)	2.15	0.45 (0.78)	0.33	0.45 (2.21)	1.76	0.45 (0.663)	0.213
7.5	0.45 (2.60)	2.15	0.45 (0.78)	0.33	0.45 (1.82)	1.37	0.45 (0.546)	0.096

<sup>1</sup>The amounts of N for N3 and N4 were reduced (in relation to N1 and N2) in 7 %, 15 % and 30 % for 2.5, 5.0 and 7.5 dS m<sup>-1</sup>, respectively.

The application of N and K (120 kg ha<sup>-1</sup> of K<sub>2</sub>O) in each treatment was distributed during the vegetative growth stage, as follows: 15 % at sowing; 25 % 20 days after sowing (DAS); 30 % 35 DAS; and 30 % 50 DAS. The reduction in N application according to ET quantities started at 20 DAS. N was applied just after an irrigation event to reduce the loss by volatilization. The other nutrients were applied following technical recommendations for maize in California, including 2.4 and 1.5 g per column of triple superphosphate (85 kg ha<sup>-1</sup> of P<sub>2</sub>O<sub>5</sub>) and a micronutrients mixture (1.0 % Zn, 2.5 % Mn, 17 % Fe, 0.1 % B, 1.0 % Cu, 0.05 % Mo, 6.0 % Ca, 3.0 % Mg and 12 % S), respectively.

Five seeds of maize (*Zea mays* L.) cv Nothstine Dent OG Lot # 41629 (Johnny's Selected Seeds, Winslow, ME, USA) were sown per column. Thinning was performed 7 DAS, leaving only one plant per column. The treatments with saline waters were initiated 8 DAS. All evaluations were carried out during the vegetative growth stage and at the beginning of the reproductive stage.

### Plant growth

The plants were collected 74 DAS, and total leaf area and dry mass production of roots, shoots (composed of leaf blades and culms plus leaf sheaths) and reproductive parts (tassel and ear) were measured. All materials were dried in an oven at 48 °C for 7 days. For total dry matter (DM), a small portion of dead material was also included.

### Leaf gas exchange and chlorophyll index

At 30, 45 and 60 days after the start of saline treatments, the leaf gas exchanges (*A* – net photosynthesis, *g<sub>s</sub>* – stomatal conductance and *E* – transpiration) were measured in

the youngest mature leaf, using an infrared gas analyzer (LI6400XT; Licor, Lincoln, NE, USA). The measurements were made between 10 and 12 AM, using an artificial source of radiation (PAR of 1800 μmol m<sup>-2</sup> s<sup>-1</sup>) and CO<sub>2</sub> concentration of 400 μmol mol<sup>-1</sup>. The chlorophyll index was measured on the same dates and in the same leaves, using a portable chlorophyll meter (Spad 502 plus; Konica/Minolta, Ramsey, NJ, USA).

### Nitrogen compounds and carbohydrates

Freeze-dried, ground and mature leaves (leaf blades) were used for the determination of total N, nitrate, organic N compounds (amino acids and protein) and carbohydrates (glucose, fructose, sucrose and starch).

Total nitrogen concentration was determined on an elemental analyzer (vario PYRO cube; Elementar Americas Inc., Mount Laurel, NJ, USA), with each sample analysed in triplicate. The extract used for the determination of nitrate and amino acids was prepared with 100 mg of freeze-dried leaf material and 10 ml of distilled deionized water. The samples were kept in a water bath for 1 h at 45 °C, mixing with a vortex at 15-min intervals, and then centrifuged for 15 min at 3000 g and stored at –20 °C. Nitrate was quantified colorimetrically by nitration of salicylic acid as described by Cataldo et al. (1975), and amino acids were quantified using the method of Yemm and Cooking (1955).

The extraction of protein was performed according to Jones et al. (1989), using ground freeze-dried samples (100 mg) and adding 8 ml of 0.1 N NaOH (pH 12.8). Protein concentration was quantified by the Bradford method (Bradford 1976) using bovine serum albumin as a standard.

For sugar determination, ground samples (100 mg) were weighed and extracted in 4 ml 80 % (v/v) ethanol in a

water bath set at 80 °C, with shaking for 30 min. The extracts were agitated for 5 s using a vortex mixer and centrifuged for 7 min at ~1000 g using a bench centrifuge (HN-SII; IEC, Needham Heights, MA, USA). The ethanol supernatant was decanted, and the samples were re-extracted three more times as described above. The pooled supernatants were tested for sugars (glucose, fructose, and sucrose) based on procedures described by Hendrix (1993). The residues from sugar extraction were used for starch determination according to Hendrix (1993) and Liu et al. (1999).

### Water- and N-use efficiency

The WUE was estimated using the following ratios: total dry mass/evapotranspiration (Total DM/ET),  $A/E$ , and carbon isotopic discrimination ( $\delta^{13}C$ ). The N-use efficiency was estimated using the following ratios: total dry mass/total N applied (Total DM/Napp),  $A/\text{total N applied}$  ( $A/N_{app}$ ) and  $A/\text{leaf N concentration}$  ( $A/N_{leaf}$ ). The  $\delta^{13}C$  were processed in triplicates using an elemental analyser (vario PYRO cube) coupled to a isotope ratio mass spectrometer (Isoprime Ltd, Cheadle, UK) and calculated according to Farquhar et al. (1982).

### Electrical conductivity and $\Delta N$ -nitrate in the soil

Four soil samples per column, of different layers (0–20, 20–40, 40–60 and 60–80 cm), were collected before and at the end of the experiment. The electrical conductivity ( $EC_e$ ) and nitrate concentration were determined in the saturation extract. The  $\Delta N$ -nitrate in the soil was obtained by the difference in the amount of nitrate in soil column between two sampling times.

### Statistical analysis

Differences among salt treatments, N application and the interaction salt  $\times$  N were tested using a two-way analysis of variance ( $F$  test). The regression analysis and Tukey's test were used to evaluate the effects of salinity and N application, respectively.

## Results

### Plant growth

According to the analysis of variance, salinity (S) affected all growth parameters evaluated ( $P < 0.01$ ), while nitrogen and the interaction S  $\times$  N affected the reproductive DM ( $P < 0.01$ ), total shoot ( $P < 0.01$ ) and total DM ( $P < 0.05$ ). The interaction between salinity and nitrogen on plant growth (Fig. 1) indicates that the beneficial effects

of high levels of N were obvious for reproductive DM (Fig. 1a), especially when plants were subjected to salt concentration up to 5.0 dS m<sup>-1</sup>. For shoots DM (Fig. 1b) and total DM (Fig. 1c), no positive effect of N was observed when salinity in the irrigation water was higher than 2.5 dS m<sup>-1</sup>. On the other hand, the treatment with N application based on the reduced ET (N3 and N4) showed similar or higher DM in relation to the N levels previously established (N1 and N2). Root growth (Fig. 1d) was also inhibited by salinity, but in this case, the decrease was not related to N rates or to the S  $\times$  N interaction.

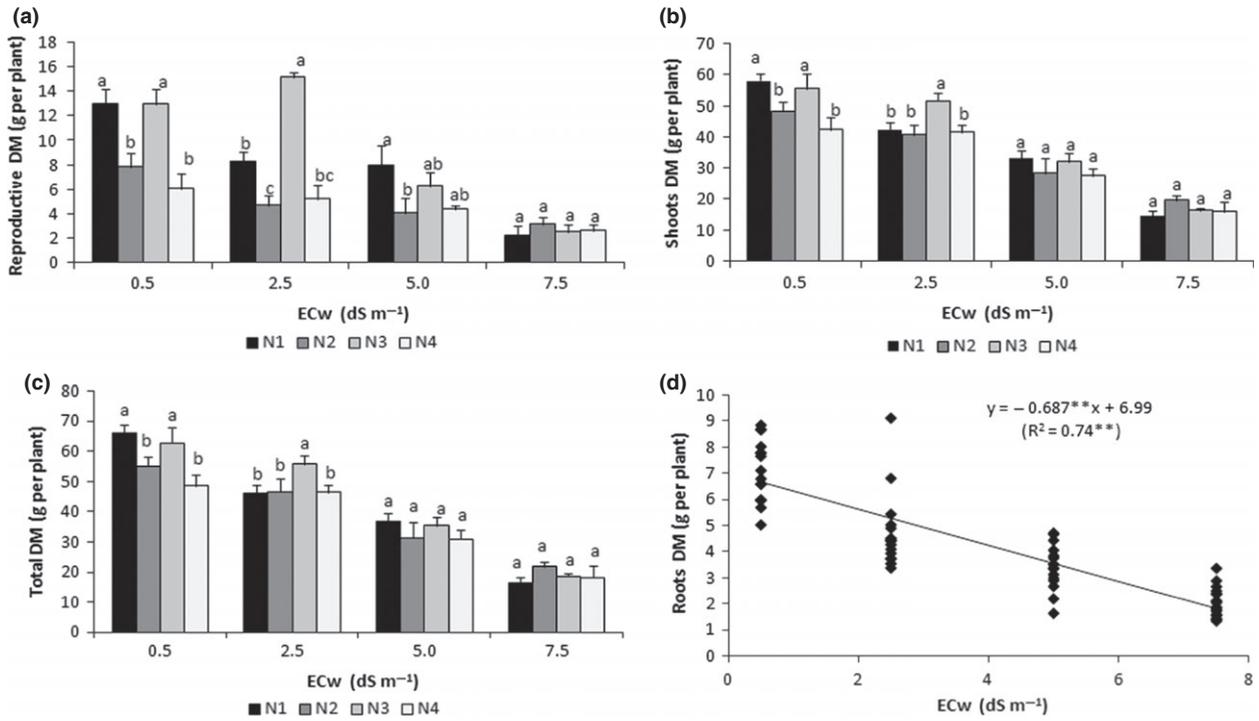
### Chlorophyll index and leaf gas exchange

Salinity, N and S  $\times$  N interaction were all significant for chlorophyll index and for all leaf gas exchange parameters ( $P < 0.01$ ). For the chlorophyll index, the effect of high levels of nitrogen was significant for plants subjected to salt concentrations up to 5.0 dS m<sup>-1</sup> (Fig. 2a). Plants under the highest level of salinity (7.5 dS m<sup>-1</sup>) showed lower values of chlorophyll index than control plants (0.5 dS m<sup>-1</sup>) and did not respond to different rates of N. It is important to emphasize that the decrease in N application based on ET (N3 and N4) did not affect chlorophyll index in relation to the N levels previously established (N1 and N2).

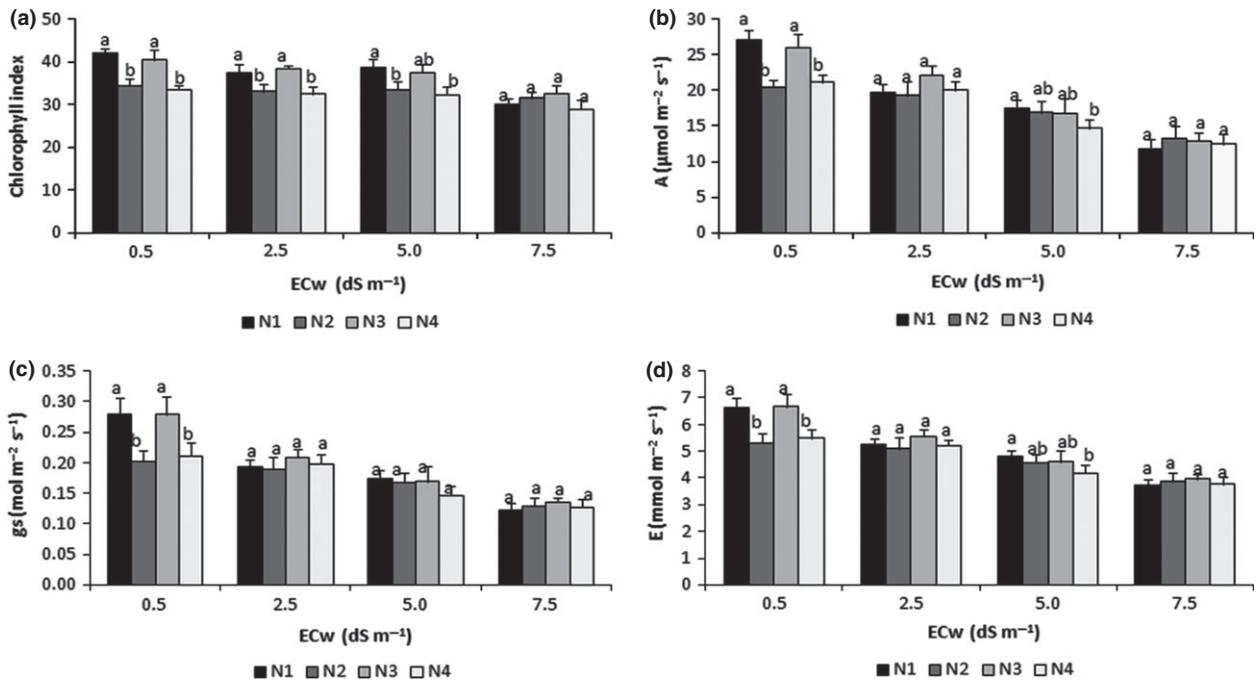
Consistent and significant effects of N rates on leaf gas exchange were observed only for control plants (Fig. 2), differing from those results observed for chlorophyll index. We observed a trend for decreasing  $A$  (Fig. 2b),  $g_s$  (Fig. 2c) and  $E$  (Fig. 2d) with increased salinity, regardless of the rate of nitrogen application. However, the decrease in N application according to ET (N3 and N4) did not affect the leaf gas exchange, regardless of salinity, in relation to the N levels previously established (N1 and N2).

### Nitrogen compounds and carbohydrates

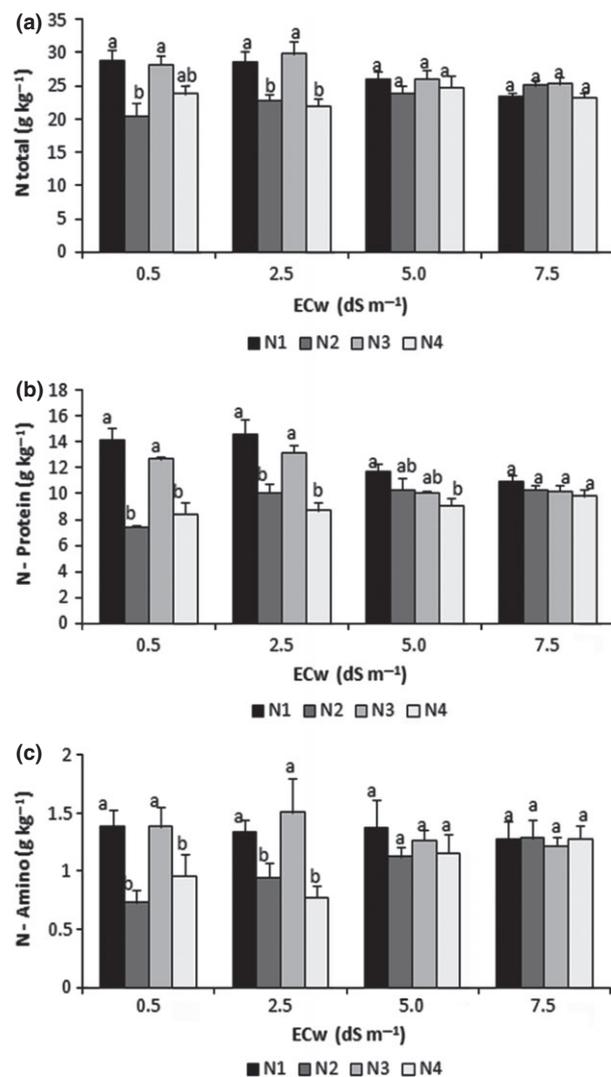
Salinity, N rates and S  $\times$  N interaction did not affect leaf nitrate concentration ( $P > 0.05$ ), and N-nitrate represented <2 % of the total nitrogen in the leaves. However, salinity, N and S  $\times$  N interaction had significant effects on leaf N concentration, protein and free amino acids ( $P < 0.01$ ). For these compounds, the effect of different rates of nitrogen was significant and consistent only for plants under salt concentration up to 2.5 dS m<sup>-1</sup> (Fig. 3). The increase in salt concentration decreased N (Fig. 3a), protein (Fig. 3b) and amino acids (Fig. 3c) in leaves when high levels of nitrogen were applied (N1 and N3). For example, for N1 rate the protein concentration decreased by 22 % in plants irrigated with water of 7.5 dS m<sup>-1</sup>, compared to control. On the other hand, increased salinity increased or did not change the leaf concentration of N compounds when low levels of N



**Fig. 1** (a) Reproductive, (b) shoots, (c) total and (d) root dry matter (DM) of maize irrigated with saline water and under different rates of nitrogen. Vertical bars represent standard errors ( $n = 4$ ). Bars with same letter, for each salt treatment, do not differ according to Tukey's test ( $P > 0.05$ ). For (d), \*\*Significance at 1 % by the  $F$  test, and we plotted all N levels together at each salinity level because there was no effect of N on root DM nor any interaction between N and salinity.



**Fig. 2** (a) Chlorophyll index, (b) net photosynthesis rate- $A$ , (c) stomatal conductance- $g_s$  and (d) rate of transpiration- $E$  of maize irrigated with saline water and under different rates of nitrogen. Vertical bars represent standard errors ( $n = 5$ ). Bars with same letter, for each salt treatment, do not differ according to Tukey's test ( $P > 0.05$ ).



**Fig. 3** (a) Leaf concentrations of total N, (b) N-protein and (c) N-amino acids of maize irrigated with saline water and under different rates of nitrogen. Vertical bars represent standard errors ( $n = 5$ ). Bars with same letter, for each salt treatment, do not differ according to Tukey's test ( $P > 0.05$ ).

EC (dS m <sup>-1</sup> )	Glu + Fru g kg <sup>-1</sup> DW	Sucrose	Total sugar	Starch
0.5	10.7ab ± 1.7	52.2ab ± 5.6	62.8ab ± 5.7	16.8a ± 3.5
2.5	13.0a ± 1.6	56.9a ± 5.6	69.9a ± 6.1	17.0a ± 2.5
5.0	11.6ab ± 1.2	52.1ab ± 7.9	63.6ab ± 7.6	17.4a ± 2.4
7.5	9.2b ± 0.9	40.7b ± 7.1	49.9b ± 8.6	14.2a ± 2.2
F test	**	*	**	ns

\*\* , \*Significant at 1 % and 5 % by *F* test, respectively; ns, non-significant. Average ± standard errors ( $n = 20$ ). Mean values with same letter, in the columns, do not differ according to Tukey's test ( $P > 0.05$ ).

were used (N2 and N4). For example, leaf protein increased 38 % in N2 plants irrigated with salinity level of 7.5 dS m<sup>-1</sup>, compared to control.

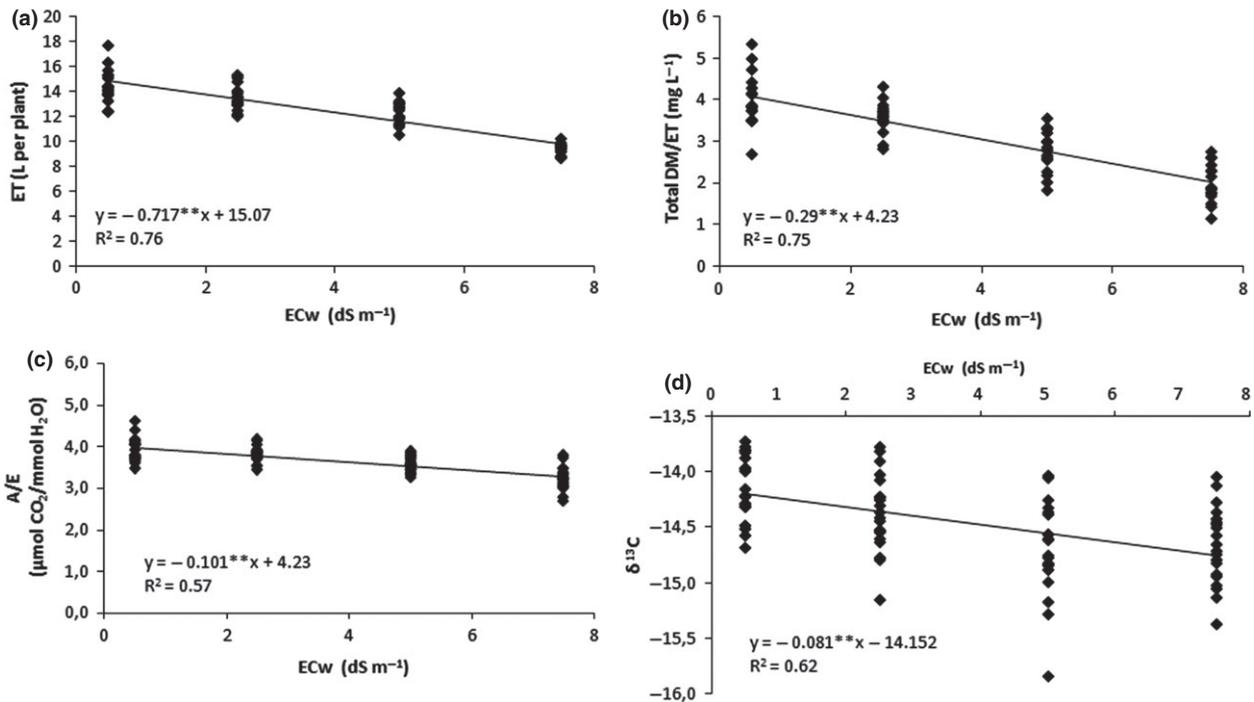
The concentrations of monosaccharide (glucose + fructose), sucrose and total sugar were affected only by salinity treatments (Table 2). For these analyses, lower values were observed in the treatments with the highest salinity level, while no difference was observed for EC<sub>w</sub> up to 5.0 dS m<sup>-1</sup> (Table 2). On the other hand, salinity and N rates did not affect starch concentration in the leaves.

### Water- and nitrogen-use efficiency

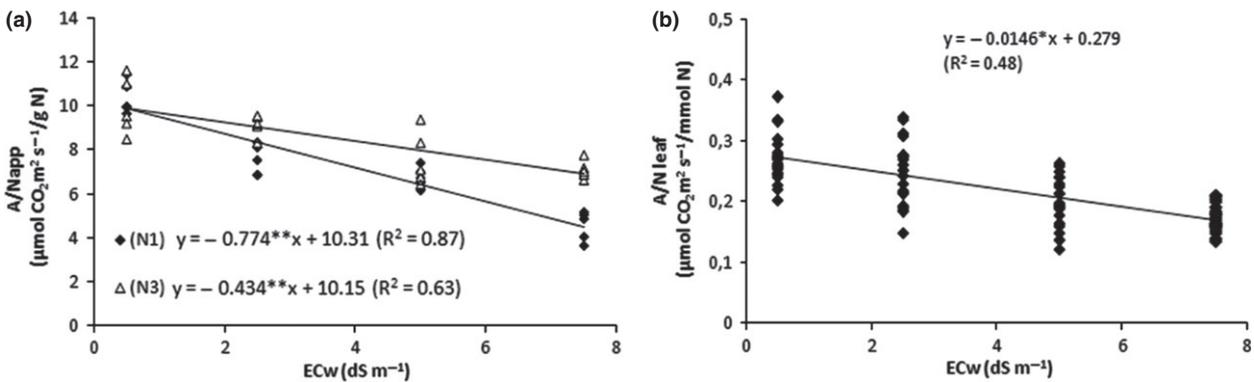
Evapotranspiration and the WUE, measured by the relationship total DM/ET, *A/E* and δ<sup>13</sup>C, were significantly affected by salt stress ( $P < 0.01$ ), but no effect of nitrogen and *S* × *N* interaction was observed. The increase in salinity caused a linear decrease in ET (Fig. 4a), total DM/ET (Fig. 4b), *A/E* (Fig. 4c) and δ<sup>13</sup>C (Fig. 4d).

The NUE measured by *A/N*leaf ratio was affected only by salinity, while salinity, N and *S* × *N* interaction had significant effects on NUE ( $P < 0.01$ ), measured by *A/N*app. The values for *A/N*app were higher for the low rates of N (N2 and N4), regardless of the level of salinity, reaching values higher than 15 μmol CO<sub>2</sub> m<sup>-2</sup> s<sup>-1</sup> per g N (data not shown). However, the decrease in the values of this parameter with the increase in salt stress was consistently higher for the N levels previously established (N1 and N2) than for treatments with lower N application based on the reduced ET (N3 and N4). For example, the linear slope for *A/N*app corresponding to N1 rate (0.774) was almost twice that of the slope calculated for N3 rate (0.434) (Fig. 5a). The same trend was observed for N4 compared to N2 (data not shown). On the other hand, the *A/N*leaf ratio decreased linearly with the increase in salinity, independent of the N rate (Fig. 5b), reaching a value 45 % lower in the highest level of salinity. Similar results were observed for total DM/*N*app and total DM/*N*leaf (data not shown).

**Table 2** Leaf concentrations of glucose + fructose, sucrose, total sugar and starch of maize irrigated with saline water



**Fig. 4** (a) Evapotranspiration (ET), (b) ratio of total dry mass to evapotranspiration-total dry matter/ET, (c) photosynthesis/transpiration ratio-A/E and (d) carbon isotopic discrimination- $\delta^{13}\text{C}$  of maize irrigated with saline water and under different rates of nitrogen. \*\*Significance at 1 % by the *F* test.

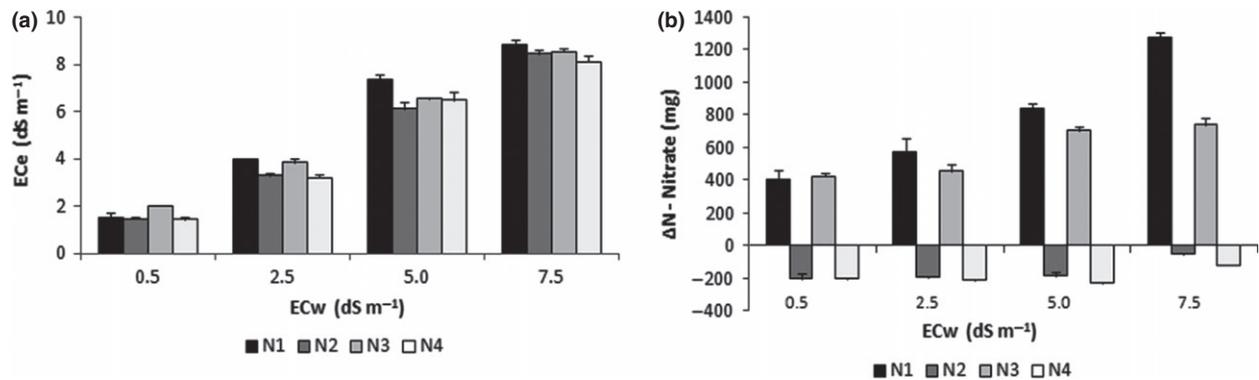


**Fig. 5** (a) Ratio of photosynthesis to total N applied-A/Napp and (b) photosynthesis to leaf N concentration ratio-A/Nleaf of maize irrigated with saline water and under different rates of nitrogen. \*\*Significance at 1 % by the *F* test.

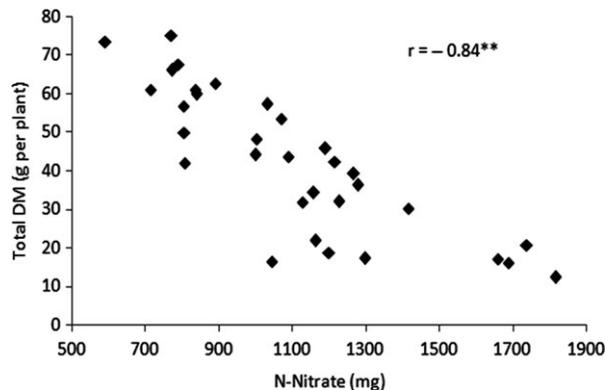
**Salt accumulation and  $\Delta\text{N}$ -nitrate in the soil**

The averaged electrical conductivity of the soil column ( $\text{EC}_e$ ) increased with the salinity of the irrigation water, but for the higher levels of salinity (5.0 and 7.5  $\text{dS m}^{-1}$ ), the  $\text{EC}_e$  values were higher when high rate of N (N1) was applied (Fig. 6a). On the other hand, the  $\Delta\text{N}$ -nitrate in the soil was consistently and significantly affected by salinity, N and  $\text{S} \times \text{N}$  interaction ( $P < 0.01$ ).

For the high levels of N (N1 and N3), an accumulation of N-nitrate was observed in the soil column at the end of the experiment, especially at high levels of salinity (Fig. 6b). For example, for N1 N-nitrate accumulation in the soil was 322 % higher in the treatments with  $\text{EC}_w$  of 7.5  $\text{dS m}^{-1}$ , related to the respective control. In addition, 88 % of the N-nitrate in this treatment was found below the 20 cm soil depth (data not shown). The N application based on ET (N3) decreased 20 %, 16 % and 42 % the



**Fig. 6** (a) Soil salt accumulation and (b)  $\Delta\text{N-nitrate}$  in the soil columns as a function of the salinity of irrigation water and different rates of nitrogen. Vertical bars represent standard errors ( $n = 4$ ).



**Fig. 7** Inverse correlation between the amount of N-nitrate per column at the end of the experiment and total dry matter (total DM) produced by maize plants. Data from N1 and N3 rates of nitrogen.  $^{**}$ Significance at 1 % by the  $t$  test

nitrate accumulation in soil columns subjected to salinities of 2.5, 5.0 and  $7.5 \text{ dS m}^{-1}$ , respectively, compared to same salt levels and N1 rate.

Negative  $\Delta\text{N-nitrate}$  was observed for all salt treatments when low rates of nitrogen (N2 and N4) were used. However, the values were also higher (close to zero) in the treatment with the highest salinity level (Fig. 6b). In addition, N application according to ET (N4) caused a more negative  $\Delta\text{N-nitrate}$  compared to N2, especially when soil columns received saline water with  $\text{EC}_w$  of 5.0 and  $7.5 \text{ dS m}^{-1}$ . There was also a negative correlation between N-nitrate in the soil column and plant growth, for N2 and N4 rates ( $r = 0.64^{**}$ ) and for N1 and N3 rates of nitrogen (Fig. 7), and the amount of N-nitrate in the soil was higher than that of N added as  $\text{KNO}_3$ . A significant part of this nitrate probably was derived from urea, as the soil conditions, such as pH, were favourable for nitrification process.

## Discussion

Nitrogen is a limiting factor for plant growth in natural ecosystems (Bradley and Morris 1992) and agricultural areas (Dong et al. 2012), and N fertilization has been used to increase plant yield under different soil conditions (Hu and Schmidhalter 2005, Hou et al. 2009, Dong et al. 2012, Razzaghi et al. 2012). However, under semi-arid climates, plant growth can be limited by different abiotic factors acting simultaneously, such as nitrogen deficiency, drought and salinity. In such cases, plants respond to interaction and feedback mechanisms between these factors (Wang and Baerenklau 2014). Thus, manipulating a single factor usually is not the solution considering that plants respond primarily to the most yield-limiting factor (Semiz et al. 2014).

In our work, the significant inhibition of growth in maize, caused by the interaction between salinity and N rates (Fig. 1), clearly shows that the osmotic and toxic effects caused by salt stress neutralizes any potential benefit from the high N rate. These results refute the hypothesis that additional N fertilization increases salt tolerance in maize. This hypothesis was also refuted for tomato (Mori et al. 2008) and lemon (Gimeno et al. 2009), based on plant growth and yield. However, the responses to N fertilization can be related to salt tolerance of the species or cultivar, that is increase in plant growth can be observed in genotypes that are more salt tolerant. Studies with halophytes (Naidoo 2009, Yuan et al. 2010, Jiang et al. 2012) and with different cultivars of olive (Tabatabaei 2006) and wheat (Nasraoui et al. 2013) agree with this hypothesis. For the halophyte *Sueda salsa* (Amaranthaceae), for example, the increase in N concentration from 1 to 10 mM increases shoot dry mass for NaCl concentrations up to 300 mM (Jiang et al. 2012).

The benefits of N application were greater for maize reproductive than for vegetative growth (Fig. 1). In plants

irrigated with  $7.5 \text{ dS m}^{-1}$ , about 14 % of DM was partitioned to reproductive parts, regardless of the rate of N application. This percentage is similar to other salinity levels, but only when low rates of N (N2 and N4) were used. For the high rates of N (N1 and N3), the reproductive part corresponded to 20 %, 22 % and 19 % of the total DM, for plants growing under 0.5, 2.5 and  $5.0 \text{ dS m}^{-1}$ , respectively. This result suggests that the pool of organic reserves, especially N compounds, can be allocated preferentially for plant reproduction under low and moderate salinity, only when N is not the limiting factor.

The response for photosynthetic rate ( $A$ ), stomatal conductance ( $g_s$ ) and transpiration ( $E$ ) were very similar for both salinity and N (Fig. 2). This similarity indicates that the decrease in  $A$  and  $E$  can be explained by stomatal closure (Munns and Tester 2008), suggesting that osmotic effects prevailed over effects related to nutritional status, especially when plants were subjected to saline treatments. For control plants, the reduction in leaf gas exchange for N2 and N4 treatments can be explained, at least in part, by the reduction in the concentration of N compounds, especially for N-protein and chlorophyll, as suggested by Naidoo (2009). In control plants that received N2 rate, for example, the N-protein represented only 38 % of the total N in the leaves, while this value for N1 was about 50 %.

Our results with maize are in agreement with those of Tabatabaei (2006) with olive, as he showed that increasing N in the nutrient solution had no effect on  $A$  in various salinity levels in salt-tolerant olive cultivars (Mission and Manzanillo), but the value of  $A$  was reduced as N level increased in the salt-sensitive cultivar (Zard). In contrast, Akram et al. (2011) showed increases in  $A$  for hybrid maize (Pioneer 32B33) grown under 5 and  $10 \text{ dS m}^{-1}$  ( $EC_e$ ) when the N rate increased from 175 to  $275 \text{ kg ha}^{-1}$ . However, these increases were not accompanied by an increase in  $g_s$ , and the values reported for  $A$  were higher than normally expected ( $A$  up to  $99.6 \mu\text{mol m}^{-2} \text{ s}^{-1}$ , under PAR up to  $1030 \mu\text{mol m}^{-2} \text{ s}^{-1}$ ).

The importance of our results is that the decrease in N application, based on ET (N3 rate), did not affect plant growth, leaf gas exchange, concentration of N compounds, carbohydrates and relative index of chlorophyll for plants subjected to different salt levels, compared to N1 rate and same levels of salinity. Plants under N3 rate were also in the same developmental stage as plants under N1 rate, based on reproductive growth evaluation at the end of the experiment. Moreover, based on physiological responses and nutritional status, one can conclude that plants under N1 and N3 had the same potential for final yield. On the other hand, rates N2 and N4 did not meet the N demand of maize. Plants under these rates, mainly at low levels of salinity (control and  $2.5 \text{ dS m}^{-1}$ ), presented typical visual symptoms of N deficiency, delayed plant development and

leaves with low levels of N compounds (protein, amino acids and chlorophyll).

The inhibition of  $\text{NO}_3^-$  uptake by  $\text{Cl}^-$  was suggested as an important effect of salinity, which can affect nutritional N status and cause inhibition in physiological processes and plant growth (Hu and Schmidhalter 2005). However, according to other authors, reductions in  $\text{NO}_3^-$  uptake appear to be better related to reduced water use than to chloride antagonism from salt stress (Lea-Cox and Syvertsen 1993, Shenker et al. 2003, Abdelgadir et al. 2005). Our data had a high positive correlation between ET and total DM ( $r = 0.94^{**}$ ) and ET and total N per plant ( $r = 0.88^{**}$ ), similar to the data obtained by other authors (Katerji et al. 2001, Shenker et al. 2003, Feng et al. 2005, Ramos et al. 2012, Wang et al. 2012). In addition, increased  $\text{Cl}^-$  concentration in irrigation water did not affect leaf N concentration when low amounts of N (N2 and N4) were added to the soil. Also, a greater availability of  $\text{NO}_3^-$  in the soil (Figs 6 & 7) did not change the leaf nitrogen concentration in plants under 5 and  $7.5 \text{ dS m}^{-1}$  (Fig. 3). However, according to Hu and Schmidhalter (2005), salinity reduces leaf  $\text{NO}_3^-$  concentration without affecting the total N content. The significance of this to salt tolerance is not clear.

Salinity caused a linear reduction in WUE, measured by instantaneous ( $A/E$ ) and more integrated methods ( $\delta^{13}\text{C}$  and total DM/ET), indicating that the effects on plant growth and leaf gas exchange were higher than those observed for water consumption (Fig. 4). The lack of effect of nitrogen rates on WUE is in agreement with Shenker et al. (2003). According to these authors, moderate N deficiency, similar to treatment used in our study, does not affect WUE in maize plants.

The reported higher values of NUE for low rates of N (N2 and N4) could lead to a wrong interpretation if the data of growth and physiological responses were not available, as they indicate limited availability of N for plants, especially at low levels of salinity. When N was not limiting for maize plants (N1 and N3), the decrease in NUE, measured by  $A/\text{Napp}$  ratio, indicated that the amount of N added to the soil, especially for N1 rate, was higher than the amount needed by plants growing at elevated salt concentrations. However, the reduced N application based on ET (N3) increased NUE ( $A/\text{Napp}$ ) for plants subjected to different salt levels, as compared for N1 rates and same levels of salinity. On the other hand, salinity decreased NUE, as measured by  $A/\text{Nleaf}$  (Fig. 5b), regardless the rate of N. These results show that salt-induced inhibition in photosynthesis was more related to osmotic and toxic effects caused by salinity than to leaf nutritional status.

The negative  $\Delta\text{N-nitrate}$  in the soil (Fig. 6) confirmed that application of 30 % of the local N recommendation did not meet the demand for maize plants, mainly when the value of  $EC_w$  was up to  $5.0 \text{ dS m}^{-1}$ . For treatments

with positive  $\Delta N$ -nitrate, it is clear that salinity decreased the N demand of maize. For control plants under N1, the positive  $\Delta N$ -nitrate indicated that 15 % of N applied as urea and  $KNO_3$  remained in the soil as nitrate, while for plants under 5.0 and 7.5  $dS\ m^{-1}$ , 32 % and 50 %, respectively, were retained in the soil, with 84 % and 88 % being found below 20 cm from surface. Thus, the use of high rates of N under saline condition represents a serious risk for ground water contamination, as previously reported (Feng et al. 2005, Bowman et al. 2006, Segal et al. 2010). The risks of contamination are greatly increased if the N rates added to salt-stressed plants are higher than values recommended to non-stressed plants, as suggested by others. On the contrary, our results revealed that, under salt stress, N fertilization based on the reduction in ET can be a way to reduce the possible risk of nitrogen losses and environmental contamination.

### Concluding remarks

Can the ET be used as a criterion to calculate adequate levels of N fertilization under saline conditions?

Despite contradictory reports on the effects of N supply on plant growth under salt stress, recent literature has shown that the amount of nitrogen needed by stressed plants can be reduced without impacts on potential crop yield, resulting in higher NUE and lesser risk of nitrate leaching and ground water contamination. Our results showed that salinity caused  $NO_3^-$  accumulation in the soil, but there was an inverse correlation between soil N-nitrate accumulation and plant growth. Apparently, the plant growth inhibition and stomatal closure observed were related to osmotic and toxic components of salt stress and, as a consequence, less water and less N were absorbed from the soil, causing nitrate accumulation.

Our results also showed that reduction in N application according to ET (N3 rate) did not affect growth and physiological responses (leaf gas exchange, chlorophyll index, nitrogen compounds and carbohydrates, and WUE) in maize, but increased NUE, and greatly reduced the nitrate accumulation in the soil. In addition, the total water consumption was correlated to plant growth and to total N per plant. We conclude that reduction in N application, based on reductions in ET, is a good strategy to reduce both the risk of ground water contamination by  $NO_3^-$  leaching and fertilization costs, without causing any additional damage to plant development under salt stress.

Although the use of ET as a criterion to establish N fertilization has produced interesting results, the concentration of N in the soil remained higher in salt-stressed plants, especially under 5.0 and 7.5  $dS\ m^{-1}$ , than in control plants. Our results and those of others indicate that certain traits of the genotype, such as demand for nutrients and

salt tolerance, can affect the interaction salinity  $\times$  N. Future studies with different salt-tolerant genotypes, the use of different methods to estimate the actual ET and the use of non-destructive methods for measuring crop growth or biomass (such as Normalized Difference Vegetation Index) can expand the knowledge of the interaction among salinity, nitrogen and water consumption by plants.

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