

## Salinity Tolerance of Kentucky Bluegrass as Affected by Nitrogen Fertilization

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### ABSTRACT

In most semiarid and arid areas, fresh water shortage compels managers to use low quality water sources with high salinity to irrigate turf and landscape. Recent research has noticed that management of nitrogen fertilization can alleviate salinity effects on plants. This greenhouse sand culture experiment was conducted in order to investigate morphological and physiological responses to salinity stress in Kentucky bluegrass (*Poa pratensis* L.) grown using different nitrogen sources. Three salinity levels (0, 40 and 80 mM NaCl) and three  $\text{NO}_3^-/\text{NH}_4^+$  ratios (6/0.5, 6/1 and 6/2) were applied in nutrient solutions. Under non saline conditions, higher ammonium concentration increased Turf Quality (TQ), leaf  $\text{NO}_3^-$ , proline content, Nitrate Reductase Activity (NRA), shoot and root growth. On the other hand, leaf potassium ( $\text{K}^+$ ) sodium ( $\text{Na}^+$ ) and MalonDiAldehyde (MDA) content were not affected. During the first week, the 40 mM NaCl treatment showed that the positive effects of  $\text{NH}_4^+$  on salinity tolerance were still perceptible. However, the 80 mM NaCl treatment showed that the adverse effects of high salinities were more pronounced when turf received high ammonium rate nutrient solution, as manifested by the decrease of TQ,  $\text{NO}_3^-$ , NRA,  $\text{K}^+/\text{Na}^+$  ratio, shoot and root growth and by the increase of leaf MDA content. This suggests that effects of  $\text{NO}_3^-/\text{NH}_4^+$  ratio on salt tolerance varies with salinity levels.

**Keywords:** Morphological and physiological responses,  $\text{NO}_3^-/\text{NH}_4^+$  ratio, Salt tolerance.

### INTRODUCTION

Kentucky bluegrass (*Poa pratensis* L.) is a cool season grass widely used for home lawns, sport fields and commercial landscapes in temperate climates. Salinity stress is one of the major factors limiting the use of Kentucky bluegrass in many arid and semiarid regions, where soil salt content is naturally high and precipitation is insufficient for soil leaching (Koch *et al.*, 2011). Because of the increasing global demand on the limited potable water resources, treated wastewater with high salinity is increasingly used to irrigate landscape and large turf facilities (Gill and Rainville, 1994). Excess of NaCl is the most

common cause of salt stress in plants. The detrimental effects of salinity on turfgrass growth include ionic toxicity, osmotic stress (osmotic inhibition of plant water absorption) and secondary stresses, such as nutritional disorders and oxidative stress. Plant salt tolerance is a complex phenomenon involving morphological, physiological, and biochemical processes. Many factors interact with plant salinity tolerance, such as irrigation management, humidity, temperature, light flux density, cultural practices, air pollution and soil fertility (Ahmad *et al.*, 2013).

Among the essential nutrients, nitrogen is usually the limiting growth nutrient required in larger amounts. Nitrate ( $\text{NO}_3^-$ ) and ammonium

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( $\text{NH}_4^+$ ) ions are the two dominant forms of nitrogen taken up by plants (Marschner, 1995). Several studies have indicated that nitrate and ammonium, as nitrogen sources, differently influence plant growth and development (Nasraoui-Hajaji and Gouia, 2014; Prinsi and Espen, 2015; Vazquez *et al.*, 2015). Nitrogen source used in plant nutrient solution also influences sensitivity to salt stress (Khaydarova and Beltrão, 2006; Neves *et al.*, 2006; Min *et al.*, 2014). It has been reported that rose plants are more sensitive to saline conditions when grown in nutrient solutions containing  $\text{NH}_4^+$  as the nitrogen source (Lorenzo *et al.*, 2001). Flores *et al.* (2003) reported that under saline conditions, increasing  $\text{NH}_4^+$  concentration in the nutrient  $\text{NO}_3^-/\text{NH}_4^+$  solutions decreased tomato yield. Ghanem *et al.* (2011) showed that, under non-saline conditions, the  $\text{NO}_3^-/\text{NH}_4^+$  ratio had no significant effect on shoot and root fresh weight of tomato plants; however, under saline conditions, decreasing the  $\text{NO}_3^-/\text{NH}_4^+$  ratio from 6/0.5 to 5/1.5 significantly enhanced the biomass production by 22%. Numerous experiments have revealed that use of  $\text{NH}_4^+$ , as the sole N source, exaggerated salt stress symptoms. However, the addition of some  $\text{NH}_4^+$  to the nutrient solution was beneficial to salinity tolerance (Kant *et al.*, 2007; Bybordi, 2010; Nathawat *et al.*, 2007; Ali *et al.*, 2001). The aims of this current work were: (1) To investigate whether  $\text{NO}_3^-/\text{NH}_4^+$  ratio can influence salinity tolerance of Kentucky bluegrass, and (2) To compare morphological and physiological effects of different  $\text{NO}_3^-/\text{NH}_4^+$  ratios in the nutrient solutions on Kentucky bluegrass, across a range of several salinity levels.

## MATERIALS AND METHODS

### Turfgrass Culture and Growth Condition

'Barimpala' Kentucky bluegrass (*Poa pratensis* L.) was seeded ( $20 \text{ g m}^{-2}$ ) in 15 cm diameter  $\times$  30 cm deep plastic pots filled with washed sand, during September, 2013. Plants

were grown in a greenhouse with average  $25^\circ\text{C}$  day/ $15^\circ\text{C}$  night temperatures under natural light (Average:  $800 \mu\text{mol m}^{-2} \text{ s}^{-1}$  photosynthetically active radiation, 14-hour photoperiod) at the University of Zanjan. Pots were fertigated daily with half strength Coic and Lesaint nutrient neutral solution (Coic and Lesaint, 1975), until drainage occurred from the bottom of the containers. Plants were fed with non-saline nutrient solution for 4 months prior to initiation of treatments. Turf was hand-clipped weekly at a 5 cm height.

### Treatments, Experimental Design, and Data Analysis

Half strength Coic and Lesaint nutrient solution was modified in order to obtain three  $\text{NO}_3^-/\text{NH}_4^+$  ratios (6/0.5, 6/1, and 6/2). Three salinity treatments (0, 40, and 80 mM NaCl) were applied by adding NaCl gradually (to avoid salinity shock) to the nutrient solutions during a 5 days period. Grasses were exposed to salinity and  $\text{NO}_3^-/\text{NH}_4^+$  ratios treatments for a period of 8 weeks. During this period, all measurements, except shoot and root growth determinations, were made every two weeks. First measurements were taken one day before initiation of treatments. The experiment was set out in a split plot design with four replications for each treatment. Salinity levels and  $\text{NO}_3^-/\text{NH}_4^+$  ratios treatments were in the main plots and subplots, respectively. To more accuracy of results, this study was repeated. This study was repeated with the same materials and methods and representative data has presented. The data were statistically analyzed using the analysis of variance procedure (SAS Institute, 2001). Differences between treatment means were separated by Fisher's protected least significance (LSD) test at the 0.05 probability level.

### Measurements

During treatments period, clipping yields were harvested weekly and dried at  $70^\circ\text{C}$  for 48 hours for dry weight determination.

Following the final clipping harvest after 8 weeks of salinity treatments, grass swards were harvested and divided into shoot system and root system. Each fraction was dried at 70°C for 48 hours and, then, dry mass was determined. Shoot growth was calculated based on the cumulative clipping and shoot system dry weight (Qian *et al.*, 2000).

Turf Quality (TQ) was visually rated on a scale of 1 to 9 based on color, density, and uniformity (Turgeon, 2002). Plants rated 1, were completely desiccated with a completely necrotic turf canopy. A rating of 9, represented healthy plants with dark green, turgid leaf blades, and a full turf canopy. A rating of 6 was considered the minimal acceptable TQ.

Proline content was measured according to the method of Bates (1973). A 0.1 g sample of fresh leaves was homogenized in 1.5 mL of 3% aqueous sulfosalicylic acid and the residue was removed by centrifugation at 15,000×g for 20 minutes. Then, one mL of the extract was mixed with 2 mL of acid ninhydrin (1.25 g ninhydrin warmed in 30 mL glacial acetic acid and 20 mL 6M phosphoric acid until dissolved) and 2 mL of glacial acetic acid and heated at 100°C for 1 hour. The reaction was terminated in an ice bath, then, 4 mL of toluene was added to the mixture and content of tube was stirred for 15 to 20 seconds. The chromophore was aspirated from the aqueous phase, and the absorbance was read at 520 nm. The amount of proline was determined from a standard curve.

Lipid peroxidation was measured in terms of MDA content (Dhindsa *et al.*, 1981). A 0.1 g sample of fresh leaves was homogenized in 1.5 mL of 5% trichloroacetic acid and the residue was removed by centrifugation at 15,000×g for 20 minutes. A 0.5 mL aliquot of the supernatant was mixed with one mL of 20% trichloroacetic acid containing 0.5% thiobarbituric acid. The mixture was heated at 100°C for 30 minutes, quickly cooled, and then centrifuged at 10,000×g for 10 minutes. The absorbance of the supernatant

was recorded at 532 and 600 nm. After subtracting the non-specific absorbance (600 nm), the concentration of MDA was calculated using an extinction coefficient of 155 mm<sup>-1</sup> cm<sup>-1</sup> (Heath and Parcker, 1968).

To determine K<sup>+</sup> and Na<sup>+</sup> contents, leaves were rinsed thoroughly and dried at 70°C for 2 days. Ground samples were dry-ashed at 550°C for 4 hours, mixed with hot 2M HCl, filtered, and then brought to a final volume of 50 mL with distilled water. K<sup>+</sup> and Na<sup>+</sup> contents were determined in these digests using an Eppendorf flame photometer (Chapman and Pratt, 1982).

Nitrate was colorimetrically determined according to the method of Treguer and Le Corre (1975) following diazotation of the nitrite obtained by reduction of NO<sub>3</sub><sup>-</sup> on a cadmium column. Leaf samples were dried at 70°C for 48 hours. Ground samples were transferred into tubes and 20 mL of 0.1N HCl was added. Samples were shaken for 24 hours and solutions were decanted. For diazotation, one mL sulfanilamide (10 g in 1 L 3N HCl) and one mL of N-naphthylethylenediamine dichloride (0.2 g in 1 L) were added. After 20 minutes, the test solutions were centrifuged for 3 min at 12,000×g and the absorbance of the supernatant was monitored at 540 nm. The amount of nitrate was determined from a standard curve.

*In vivo* Nitrate Reductase (NR) activity was determined by a modification of the method described by Ferrari and Varner (1970). The method is based on the determination of nitrite which is formed as product of the reduction of nitrate in the incubation medium. Briefly, Fresh leaves were cut into pieces of 5 mm length. Approximately 0.5 g of the tissue was placed in 5 mL of potassium phosphate buffer [0.1M KNO<sub>3</sub>, 0.1M KH<sub>2</sub>PO<sub>4</sub> and 1% (v/v) 1-propanol; pH: 7.5] in the test tube. The medium was flushed with nitrogen gas for 1 minute to purge oxygen. Samples were incubated in a water bath with gentle shaking at 30°C in the dark for one hour. After incubation, 1.0 mL of aliquots were taken and 0.5 mL of 1% (w/v) sulfanilamide



in 3 N HCl and 0.5 mL of 0.02% (w/v) N-(1-naphthyl)-ethylenediamine dihydrochloride were added to the samples. Absorbance of the supernatant was read at 540 nm and the concentration of nitrite was calculated from a standard calibration curve using  $\text{KNO}_2$ . The NR activity was expressed as n mol nitrite produced per gram fresh tissue per hour.

## RESULTS

### Shoot and Root Growth

Under non-saline conditions, shoot and root growth of Kentucky bluegrass were increased with the  $\text{NH}_4^+$  concentration enhance in the nutrient solutions. However, no significant differences between 6/1 and 6/2 ratios treatments were observed. Salinity reduced shoot and root growth regardless of  $\text{NO}_3^-/\text{NH}_4^+$  ratios. In 40 mM NaCl, highest shoot dry weight was found in 6/1 treatment, while 6/0.5 and 6/2 treatments were not significantly different. Lowest root growth under 40 mM NaCl was recorded in 6/2 treatment. The  $\text{NO}_3^-/\text{NH}_4^+$  ratios caused a significant decline in shoot and root growth under 80 mM NaCl (Figure 1).

only slightly affected by nitrogen source; however, 6/1 and 6/2 ratios treatments showed higher turf quality at the end of experimental period. In 40 mM NaCl treatment, turf quality in all plants was increased within the first 2 weeks of treatments. A greater decline in turf quality was observed in the 6/2 treatment. In the 80 mM NaCl treatment, turf quality slightly increased in the 6/0.5 treatment until 2 weeks, and then gradually decreased to below the initial level. A continuous and greater decline in turf quality was detected with the increasing  $\text{NH}_4^+$  concentration in the nutrient solutions (Figures 2 and 3).

### Potassium and Sodium Content

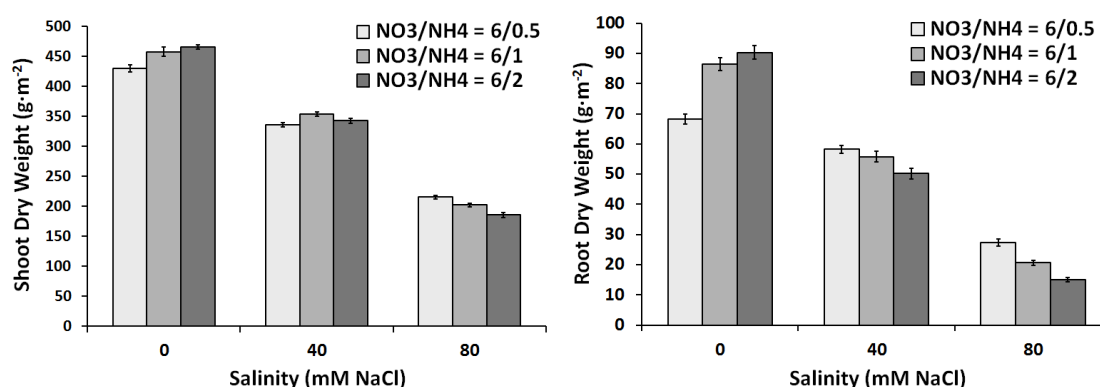
Leaf  $\text{Na}^+$  and  $\text{K}^+$  contents of non-stressed plants remained fairly constant over time. The increasing salinity and the progression of salt stress increased  $\text{Na}^+$  and decreased  $\text{K}^+$  in leaf in all plants. After 4 weeks of salt stress, turf from 6/2 treatment accumulated more  $\text{Na}^+$  and less  $\text{K}^+$  contents than the other nitrogen treatments, while no significant differences between 6/0.5 and 6/1 treatments were detected (Figure 4).

### Proline Content

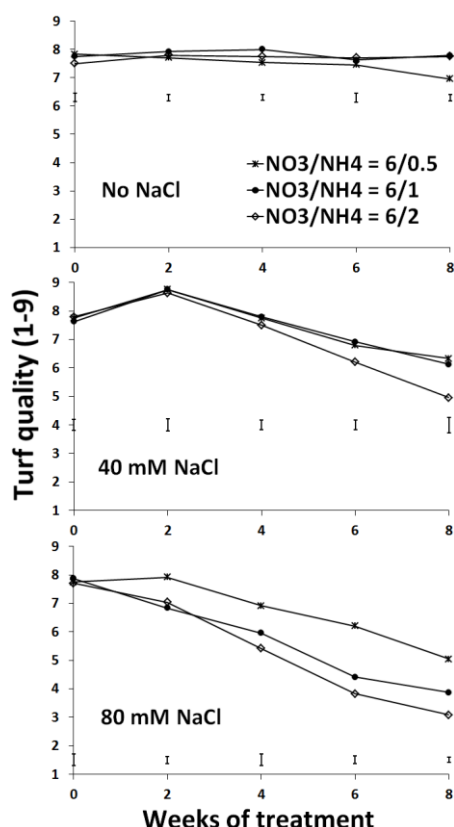
Leaf proline content increased with increasing salinity and progression of stress.

### Turf Quality (TQ)

In the non-saline treatment, plants were



**Figure 1.** Effects of nitrate/ammonium ratio and salinity on shoot and root growth of 'Barimpala' Kentucky bluegrass. Vertical bars indicate standard errors.



**Figure 2.** Effects of nitrate/ammonium ratio and salinity on turf quality of 'Barimpala' Kentucky bluegrass. Turf quality was rated 1 to 9, where 1= Poorest quality; 6= Lowest acceptable quality, and 9= Best quality. Vertical bars indicate *LSD* values ( $P \leq 0.05$ ) for treatment comparisons at a given week of treatment.

Also, in plants fed with higher rates of  $\text{NH}_4^+$ ,

more proline content was recorded. However, under non saline condition and in 40 mM NaCl, no significant differences existed in levels of proline among  $\text{NO}_3^-/\text{NH}_4^+$  ratios treatments during the first 4 weeks of experimental period (Figure 5).

### Malondialdehyde Content

Under non-saline conditions, no remarkable differences were observed in MDA content among nitrogen source treatments. In 40 mM NaCl, 6/2 treatment showed higher MDA content than 6/1 and 6/0.5 ratios treatments after 2 weeks, while 6/1 and 6/0.5 treatments were not significantly different. In 80 mM NaCl, after 2 weeks of stress, highest levels of MDA content was found in 6/2 treatment, followed by 6/1 and 6/0.5 treatments (Figure 5).

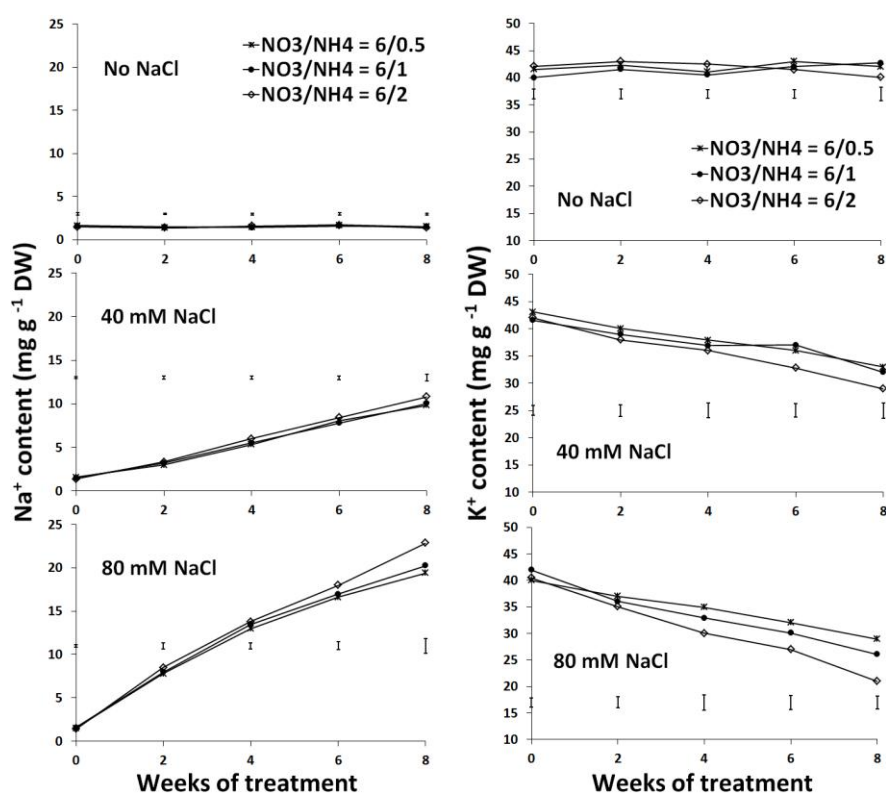
### $\text{KNO}_3$ Content and Nitrate Reductase Activity

In the absence of salt, highest levels of  $\text{NO}_3^-$  content were recorded in 6/2 treatment, followed by 6/1 and 6/0.5 treatments. Salt stress reduced  $\text{NO}_3^-$  concentration in all plants. under 40 mM NaCl, leaf  $\text{KNO}_3^-$  content in 6/0.5 treatment was significantly lower than 6/1 and 6/2 treatments after 2 weeks, while no significant differences were found between 6/1 and 6/2 treatments. Four weeks after seedling, more  $\text{KNO}_3^-$  content

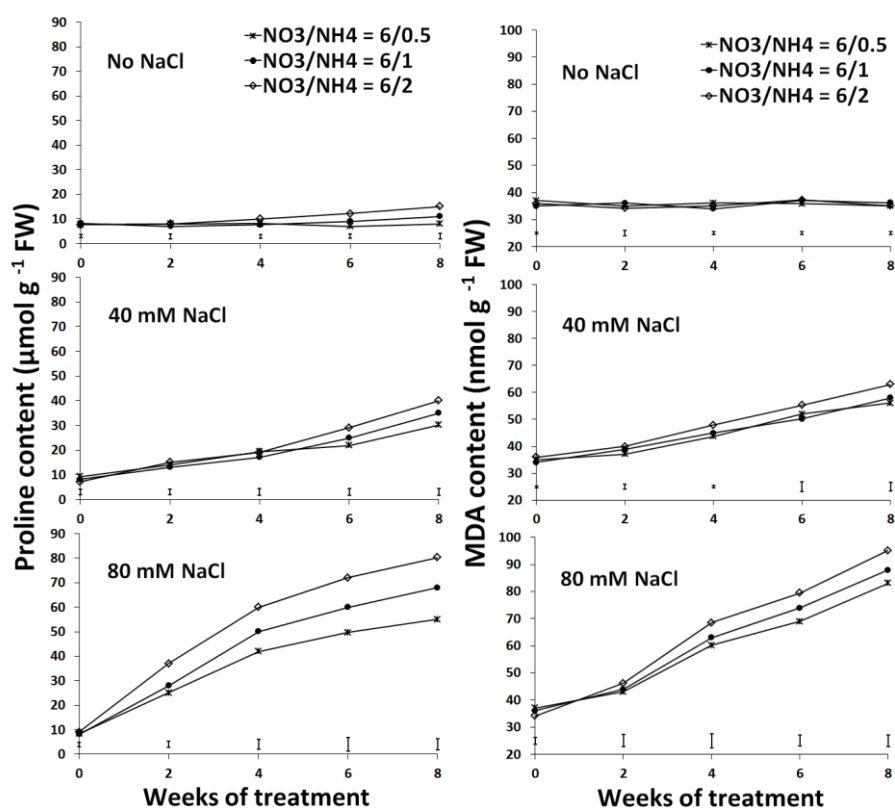


**Figure 3.** Samples of plants, showing turf quality rating: (a) Lowest quality, rating 1; (b) Medium quality, rating 5, and (c) Best quality, rating 9.





**Figure 4.** Effects of nitrate/ammonium ratio and salinity on sodium and potassium content of 'Barimpala' Kentucky bluegrass. Vertical bars indicate *LSD* values ( $P \leq 0.05$ ) for treatment comparisons at a given week of treatment.



**Figure 5.** Effects of nitrate/ammonium ratio and salinity on proline and MalonDiAldehyde (MDA) content of 'Barimpala' Kentucky bluegrass. Vertical bars indicate *LSD* values ( $P \leq 0.05$ ) for treatment comparisons at a given week of treatment.

was detected in the 80 mM NaCl, 6/1 and 6/0.5 treatments than in the 6/2 treatment. A similar pattern was observed for Nitrate Reductase Activity (NRA), except in 40 mM NaCl treatment, where a low increase in NRA activity was detected during the first 2 weeks and nitrogen treatments were not significantly different until week 8 (Figure 6).

## DISCUSSION

It has been shown that nutrient-salt interactions can affect plant growth. Fertilization can increase tolerance to salinity; however, the Electrical Conductivity of the nutrient solution (EC<sub>w</sub>) can be a limiting factor (Beltrão *et al.*, 2014). At high salinity levels, the osmotic effect is more pronounced and enhanced fertilization may provoke a negative effect on crop yield (Beltrão *et al.*, 1993, 2002). Similarly, in our study, morphological and physiological responses of Kentucky bluegrass to nutrient solution's NO<sub>3</sub><sup>-</sup>/NH<sub>4</sub><sup>+</sup> ratio differed under salinity and non-saline condition. Feeding with ammonium can be beneficial for plant growth and quality, via its less metabolic cost of absorption and assimilation than the nitrate, increasing nitrate uptake and rhizosphere pH regulation (Marschner, 1995). This would explain the increase in shoot and root growth of non-salinity stressed plants with the increase in ammonium observed in our study. However, plants that received higher amount of ammonium were more sensitive to saline conditions. Lewis *et al.* (1989) suggested that ammonium made plants more susceptible to salinity stress. This aspect is due to the fact that the assimilation of NH<sub>4</sub><sup>+</sup>, that occurs predominantly in the root is curtailed under salinity, because most available energy is used for osmoregulation. Consequently, the increased root carbon skeletons consumption for ammonium assimilation will lead to a reduced carbohydrate availability and inhibit root and shoot growth.

Some studies have demonstrated that salinity inhibits the transport of nitrate from the roots to the shoots (Abd-El Baki *et al.*, 2000; Cramer *et al.*, 1995). Our results also showed that KNO<sub>3</sub> leaf content decreased with increasing salinity and progression of salt stress. In addition, salinity reduces nitrate uptake by direct competition of chloride with nitrate (Cramer *et al.*, 1985). Nitrate assimilation is required for plant growth and development. Nitrate Reductase (NR), the first enzyme in the nitrate assimilation pathway, is considered as the limiting step for conversion of nitrate to amino acids and, so, for protein synthesis (Mane *et al.*, 2011). Nitrate regulates NR transcription, translation, and activation in higher plants (Debouba *et al.*, 2007). In our study, NR depression could be related to the low nitrate availability in the salt treated grasses. However, in non-saline condition, NR activity was increased by increasing NH<sub>4</sub><sup>+</sup>. This could be due to increased nitrate uptake by synergistic effect of ammonium. This observation agrees with the report of Bybordi (2010) where NR was increased by increasing NH<sub>4</sub><sup>+</sup> to 50% and then declined at a higher ratio of ammonium.

Proline is a multifunctional amino acid and one of the most common compatible solutes or osmoprotectant. In several turfgrass species, proline accumulation has been correlated with salinity and tissue Na<sup>+</sup> concentration (Uddin *et al.*, 2012; Lu *et al.*, 2007). Similarly, our data showed that plants fed with lower rates of NH<sub>4</sub><sup>+</sup> had lower proline content that could be resulted by less Na<sup>+</sup> accumulation. Likewise, Martínez *et al.* (1994) reported that salt stressed plants fed with NO<sub>3</sub><sup>-</sup> plus NH<sub>4</sub><sup>+</sup> accumulated more proline than plants fed with only NO<sub>3</sub><sup>-</sup>.

Specific injury through Na<sup>+</sup> accumulation rather than osmotic stress was suggested to be the main reason for NaCl susceptibility. The Na<sup>+</sup> toxicity is characterized by leaf burn, necrotic spots, and limited leaf expansion (Pessarakli, 2010), which in turn directly reduces turf quality, according to our study. However, low levels of salinity and initial periods of stress increased turf



quality probably due to the inhibition of growth and improvement of leaves color. It is widely recognized that a high  $\text{Na}^+$  concentration inhibits  $\text{K}^+$  uptake by plants due to the antagonism between the two cations. On the other hand, ammonium can also inhibit the translocation of  $\text{K}^+$  (Ashraf and Sultana, 2000). Potassium is an essential nutrient and is required in large amounts for most of the biochemical and physiological processes (such as enzymatic reactions and cell turgor pressure maintenance) that influence plant growth and metabolism. Accordingly, ammonium applied in plant nutrient solution can intensify the adverse effect of high sodium concentration.

Salt stress, like other abiotic stresses induces oxidative stress, resulting from the increase in Reactive Oxygen Species (ROS) production such as superoxide ( $\text{O}_2^{\cdot-}$ ), Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radicals ( $\text{OH}^{\cdot}$ ). All these compounds react with lipids, proteins, and DNA and induce structural damage to cell membranes and macromolecules (Mittler, 2002). In the present research, dramatic increase in MalonDiAldehyde (MDA) content, a product of lipid peroxidation, which is an indicator of free radicals damage to cell membrane under stress condition (Smirnoff, 1995), was more pronounced in high ammonium fed plants. This aspect can be explained according to the finding that  $\text{NH}_4^+$  increases antioxidant enzymes activities (Polesskaya et al., 2004; Misra and Gupta, 2006), which may suggest a higher rate of ROS production in the presence of ammonium ions.

## CONCLUSIONS

As concluding remarks, it is suggested that the presence of  $\text{NH}_4^+$  in nutrient solution was beneficial for Kentucky bluegrass growth and quality under non-saline conditions or low salinity levels. However, under severe salinity stress, high rate of ammonium application reduces salt tolerance. Declining salt tolerance due to

ammonium ion could be related to effects of  $\text{NH}_4^+$  on the decreased  $\text{K}^+$  content and, consequently, lower  $\text{K}^+/\text{Na}^+$  ratio, as well as higher rate of Reactive Oxygen Species (ROS) production, as manifested by MDA accumulation under stress condition. Therefore, additional studies are required to find proper ammonium application rates for various turfgrass species and cultivars at different salinity levels.

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## تأثیر تغذیه نیتروژن بر تحمل به شوری چمن فریز

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### چکیده

در بیشتر مناطق خشک و نیمه خشک، کمبود آب شرب مدیران را مجبور به استفاده از آبهای بی کیفیت، با شوری بالا، برای آبیاری چمن و فضای سبز نموده است. پژوهشهای اخیر نشان داده است که

مدیریت تغذیه نیتروژن می تواند اثرات مضر تنش شوری بر گیاهان را کاهش دهد. این پژوهش گلخانه ای به صورت هیدروپونیک با بستر کاشت ماسه و به منظور مطالعه پاسخهای فیزیولوژیکی و مورفولوژیکی چمن فریژ (*Poa pratensis* L.) به تنش شوری تحت شرایط منابع مختلف نیتروژن صورت گرفت. سه سطح شوری (۰، ۴۰ و ۸۰ میلی مولار کلرید سدیم) و سه سطح نسبت نیترات به آمونیوم (۶/۰.۵، ۶/۱، ۶/۲) در محلولهای غذایی اعمال شد. در شرایط بدون تنش افزایش غلظت آمونیوم کیفیت چمن، رشد ریشه و شاخساره، میزان نیترات، پرولین و فعالیت آنزیم نیترات ردوکتاز برگها را افزایش داد، در حالی که میزان سدیم، پتاسیم و مالون دی آلدهاید برگها تحت تاثیر قرار نگرفت. در شوری ۴۰ میلی مولار کلرید سدیم و در هفته های ابتدایی اجرای تیمارها، اثرات مثبت آمونیوم بر تحمل به شوری گیاهان همچنان قابل مشاهده بود، ولی در شوری ۸۰ میلی مولار کلرید سدیم اثرات مضر سطوح بالای نمک در چمنهای تغذیه شده با غلظت زیاد آمونیوم تشدید شد و کاهش کیفیت ظاهری، رشد شاخساره، رشد ریشه، نسبت پتاسیم به سدیم، میزان نیترات و فعالیت آنزیم نیترات ردوکتاز و همچنین افزایش میزان مالون دی آلدهاید برگها بیان گر این موضوع است. نتایج این پژوهش نشان می دهد که اثر نسبت نیترات به آمونیوم محلول غذایی بر تحمل به شوری چمن در سطوح مختلف نمک متفاوت است.