Moderately low nitrogen application mitigate the negative effects of salt stress on annual ryegrass

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Appropriate application of nitrogen (N) can alleviate the damage of plants caused by salt stress. This study tries to explore the change of nitrogen requirement under relatively low salt concentrations (50 mM, 100 mM) of feeding annual ryegrass and investigate the underlying mitigation mechanism. Results showed that low levels of salt stress decreased N requirement because the increment of plant height and biomass reached maximum at a relative low N level (2.0 mM not 5.0 mM). Under salt treatment, especially at 50 mM NaCl, the OJIP curve and a series of performance indexes (PI_{ABS}, RC/CS_0, ET_0/CS_0, ϕE_0, ϕ_0) achieved maximum whereas DI_0/RC, V_j and M_0 were the lowest under moderately low N level (2.0 mM). In addition, under salt stress, moderately low N application could maintain the expression of NR (nitrate reductase) and GS (glutamine synthetase) encoding genes at a relatively stable level but had no effect on the expression of detected NRT (nitrate transporter) gene. The seedlings cultured at 2.0 mM N also have the highest activity of CAT and POD antioxidant enzymes and the lowest MDA content and EL under relative low level of salt treatment. These results indicated that low level of salt treatment might reduce N requirement of annual ryegrass and moderately low N application could promote their growth mainly by regulating photosynthesis, alleviating the damage caused by ROS and maintaining the metabolism of N in annual ryegrass seedlings. These results also can provide useful reference for nitrogen application in moderation rather than in excess on annual ryegrass in mild or medium salinity areas through understanding the underlying response mechanisms.

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Abstract

Appropriate application of nitrogen (N) can alleviate the damage of plants caused by salt stress. This study tries to explore the change of nitrogen requirement under relatively low salt concentrations (50 mM, 100 mM) of feeding annual ryegrass and investigate the underlying mitigation mechanism. Results showed that low levels of salt stress decreased N requirement because the increment of plant height and biomass reached maximum at a relative low N level (2.0 mM not 5.0 mM). Under salt treatment, especially at 50 mM NaCl, the OJIP curve and a series of performance indexes (PI_{ABS}, RC/CS_0, ET_0/CS_0, ϕE_0, ϕ_0) achieved maximum whereas DI_0/RC, V_j and M_0 were the lowest under moderately low N level (2.0 mM). In addition, under salt stress, moderately low N application could maintain the expression of NR (nitrate reductase) and GS (glutamine synthetase) encoding genes at a relatively stable level but had no effect on the expression of detected NRT (nitrate transporter) gene. The seedlings cultured at 2.0 mM N also have the highest activity of CAT and POD antioxidant enzymes and the lowest MDA content and EL under relative low level of salt treatment. These results indicated that low level of salt treatment might reduce N requirement of annual ryegrass and moderately low N application could promote their growth mainly by regulating photosynthesis, alleviating the damage caused by ROS and maintaining the metabolism of N in annual ryegrass seedlings. These results also can provide useful reference for nitrogen application in moderation rather than in excess on annual ryegrass in mild or medium salinity areas through understanding the underlying response mechanisms.

Introduction

Feeding annual ryegrass (*Lolium multiflorum* Lam.) is considered to be an important forage grass with high yield, good palatability and high nutritive value (*Castanheira et al., 2014*). Salinity stress is one of the major factors limiting annual ryegrass growth and productivity. Studies have shown that the adverse effects of salinity on plants include ionic toxicity, osmotic stress and secondary stresses, such as lower photosynthesis, oxidative stress and nutritional disorders (*Allakhverdiev & Murata, 2004*; *Kalaji et al., 2011*; *Zhu, 2001*). The forage quality parameters, such as crude protein, organic matter could be seriously affected by elevated salinity level (*Robinson et al., 2004*). Plants have established a series of response mechanism to resist the external salt stress and reduce their damage in the long term of evolvement (*Deinlein et al., 2014*; *Zhu, 2001*). For example, in response to oxidative stress caused by salt, a series of antioxidant enzymes were induced to scavenge the production of reactive oxygen species (ROS) (*Kohler et al., 2009*; *Olmos et al., 1994*), such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT) (*Apel & Hirt, 2004*; *Dong et al., 2001*).

N is usually the limiting growth nutrient required in larger amounts which is deficient in saline environment and N application is the most commonly used effective method to regulate plant growth under salt stress. The inorganic N plants used is mainly ammonium N ($NH4^+$) and nitrate N (NO_3^-) transported by their transporters AMT (Ammonium transporter) and NRT (Nitrate transporter) respectively (*Giagnoni et al., 2015*). The inorganic N was then assimilated and converted into amino acid via several enzymes such as nitrate reductase (NR), glutamine

synthetase (GS) and glutamine 2-oxoglutarate aminotransferase (GOGAT) (*Xu et al., 2012*). The high salinity has also been shown to inhibit the activity of many enzymes such as NR and GS/GOGAT, involved in N assimilation in maize, cowpea, mung bean, tomato and rice (*Chakrabarti & Mukherji, 2007*; *Debouba et al., 2007*; *Khan & Srivastava, 1998*; *Parul et al., 2015*; *Silveira et al., 2001*; *Wang et al., 2012*), and then affect the absorption and utilization of N in plants (*Singh et al., 2016*). N use efficiency was also reported to be reduced significantly with increased salinity conditions (*Murtaza et al., 2014*; *Murtaza et al., 2013*). On the contrary, some studies showed that processes related to N uptake and assimilation were stimulated under certain levels of salt stress in some species. For example, salt can induce the expression level of nitrate transporter such as McNRT1 (*Popova et al., 2003*). The nitrate uptake rate and activity of NR upon NaCl exposure were promoted in *Salicornia europaea* (*Nie et al., 2015*). Therefore, it has been suggested that alteration of plant N nutrition level may hold great promise for regulating salinity response in different species under certain salt level (*Chen et al., 2014*).

On the whole, N application can reduce the negative influence of salinity by compensating and correcting nutritional imbalances in higher plants (*Esmaili et al., 2008*; *Gómez et al., 1996*; *Mansour, 2000*; *Villa et al., 2003*). Several N containing compounds are accumulated in plants subjected to salinity (*Dluzniewska et al., 2007*; *Ehlting et al., 2007*; *Sudmalis et al., 2018*). Accumulation of these compounds has been reported to participate in osmotic adjustment, promoting the photosynthetic capacity and mitigating oxidative stress by scavenging ROS (*Homaee et al., 2002*; *Kaur-Sawhney & Galston, 1979*; *Mansour, 2000*; *Rontein et al., 2002*; *Song et al., 2006*). Although many studies have shown that N plays an important role in the

amelioration of salt tolerance, it has been known that the alleviation of salt inhibition from N application shows a certain range. For example, in tomato, at the higher salinity levels, increasing N application was found ineffective in resisting negative influences caused by the enhanced salt concentrations (*Papadopoulos & Rendig, 1983*). Previous study reported that low levels of N can mitigate the negative effects whereas high N levels may exacerbate the adverse effects of salt stress on photosynthetic rate of chickpea leaves (*Soussi et al., 1998*) . Recent study also found that, low to moderate N application can mitigate the adverse effects, but excessive N could elevate the negative effects of salt stress on cotton growth (*Chen et al., 2010*). Some studies also pointed out that excessive nitrogen fertilization might lead to more pronounced osmotic effect and then provoke the negative effect on crop yield at high salinity levels (*Beltrão et al., 2002*). In addition, in high-salt soils, excessive application of N fertilizer will cause soil secondary salinization, which in turn increases the adverse effects of salt on crop growth *(Chen et al., 2010*). Moreover, over fertilization with N may contribute to N leaching in the salinity soil, where plants can not utilize the supplied N fertilizer efficiently and cause the pollution of soil and groundwater (*Pessarakli & Tucker, 1998*; *Shenker et al., 2003*, *Ward, 2013*).

Therefore, the requirements of N for plants in salinity environment might be different than those in normal environment probably due to the different physical and chemical properties of soils or substrate and the alteration of plants nitrogen use efficiency and other physiological response. Proper N fertilizer management in plants is necessary for different salt conditions to reach the aim of reducing the negative influence of salinity and minimize the degradation of soil and groundwater. Previous study on the annual ryegrass reported that increasing N concentration

100 in the nutrient solution enhanced shoot biomass production under relatively high salinity levels
101 (*Sagi et al., 1997*). However, at relatively low salt concentrations, how the optimize nitrogen
102 demand of annual seedlings could change and the possible mechanisms underlying this
103 alleviation are still not fully explored. Base on the above studies, the objectives of this work were
104 to assess the optimal N level under relatively low salt level and investigate the possible
105 mechanism of the N level-mediated alleviation of salt stress by analyzing physiological indexes
106 and metabolism of N in annual ryegrass seedlings.

107 **Materials and Methods**

108 **Plant materials and growth conditions**

109 Annual ryegrass seeds were firstly thowed in plastic containers filled with plant growth medium
110 and then cultured in greenhouse with natural sunlight. After one month, the seedlings were then
111 transferred into Erlenmeyer flasks containing 585mL nutrient solution. The seedlings were then
112 mowed to a height of 12.5 cm before the treatments were initiated. The experiment included
113 control (0 mM NaCl) and NaCl treatment (50 mM or 100 mM). Both control and NaCl treatment
114 included different nitrogen application level respectively (using NH_4NO_3 as nitrogen source).
115 The hydroponic culture was processed in a growth chamber under the following conditions:
116 22/18°C (day/night), 65% relative humidity, 300 μmol $m^{-2}s^{-1}$ photons and a 16-h day/8-h night
117 cycle. The culture solution was refreshed every two days.

118 **Chlorophyll *a* fluorescence transient and the JIP-Test**

119 A pulse amplitude modulation fluorometer (PAM2500, Heinz Walz GmbH) was used to detect
120 the Chlorophyll a fluorescence transient. The leaves of plants were put in dark place for 30 min,
121 the leaves were then exposed to 3,000 μmol photons m^{-2} s^{-1} red light condition. Each treatment
122 was replicated at least three times. Based on the theory of energy fluxes in biofilm, the JIP test
123 can further translate the primary data into other biophysical parameters (*Force et al., 2003*). The
124 basic parameters were then used to calculate a series of parameters (*Yusuf et al., 2010*).

125 **Chlorophyll content and electrolyte leakage**

126 SPAD 502 Plus Chlorophyll Meter (SPAD-502Plus, Spectrum Technologies, Inc., USA) was
127 used to quantify the leaf chlorophyll content. The electrolyte leakage (EL) were determined
128 according to the previous method (*Blum & Ebercon, 1981*).

129 **Enzymes activity and lipid peroxidation**

130 0.3 g of fully expanded leaves were immediately grounded into powder with liquid N. 4 mL ice-
131 cold phosphate buffer (50 mM, pH 7.8) was then added into the powder and the samples were
132 centrifuge at 12,000 rpm for 20 min at 4°C. The supernatant was collected to detect the activity
133 of POD and CAT and the content of MDA. The detection was based on the method as described
134 by previous study (*Fu & Huang, 2001*).

135 **Quantitative RT-PCR Analysis**

136 Total RNA was isolated and reverse transcribed using the RNeasy kit (Qiagen) and TaqMan

reverse transcription kit (Applied Biosystems). Quantitative real-time RT-PCR analysis was conducted using SYBR Green real-time PCR master mix (Toyobo, Japan) and ABI real-time PCR system (Applied Biosystems, FosterCity, CA). The primers used are listed in Supplemental Table S1. The ryegrass *Actin* gene was used as an inner control, and comparative Ct method was applied for analysis.

Statistical Analysis

One-way ANOVA was performed using SPSS17.0 for Windows (SPSS). All of above tests had at least three independent replicates. Results were expressed as mean ± *SD*, and letters show significant differences *(P* < 0.05) by Student's *t*–test..

Results

Effect of different N level treatment on the growth of annual ryegrass seedlings under NaCl stress

Under control condition, the plant height and the relative increase of biomass of annual ryegrass seedlings achieved maximum under 5.0 mM N, and then decreased a little under 10 mM N. As compared to 5.0 mM N, the height of seedlings grown under 2.0 mM N and 10 mM N decreased by about 10% and 15% respectively (Fig. 1A, B). However, under 50 mM or 100 mM NaCl treatment, the plant height achieved maximum under 2.0 mM N and then decreased a little under 5.0 mM N. In addition, salt treatment dramatically reduced the height of plants grown under 5.0 mM or 10 mM N compared with their respective control plants. However, under 2.0 mM N

condition, the plant height showed no significant difference with or without salt treatment (Fig. 1B). When exposed to 50 mM NaCl, the relative increase of biomass showed no significant difference among different N levels. After the plants were exposed to 100 mM NaCl for 10 days, the biomass only increased when 2.0mM N was applied whereas the biomass decreased when extra nitrogen (5.0 or 10 mM N) was applied (Fig. 1C). These similar alteration trends in biomass and plant height suggested that salt stress might change the N requirement of annual ryegrass and moderately reducing N application might alleviate the inhibition effect of salt stress on annual ryegrass seedlings growth. We then added a lower concentration (0.5 mM) of N treatment to investigate whether ultra-low N treatment had a moderating function on the growth of annual ryegrass seedlings under salt stress. The results showed that the plant height of annual ryegrass seedlings achieved maximum under 5.0 mM N without salt treatment. However, when expose to 50 mM or 100 mM NaCl, the plant height reached maximum under 2.0 mM N (Fig. 2A, B), showing the similar tendency with the above experiment (Fig. 1A, B). In addition, without NaCl treatment, there was no significant difference of plant height when seedlings were supplemented with lower N concentration (0.5 mM or 2.0 mM). However, under NaCl treatment, the plant height was significantly increased under 2.0 mM N compared with 0.5 mM N (Fig. S1). These results indicated that the alleviating effect of N application on the growth inhibition of annual ryegrass under salt stress might have a certain range. Moderately low N could alleviate the inhibition of annual ryegrass growth by salt stress through a series of response mechanism, whereas ultra-low N could not promote, but seriously inhibit the growth of annual ryegrass.

Impact of N on the OJIP transient curve in the leaves of annual ryegrass under NaCl stress

In order to understand moderate N-mediated alleviation of salt stress on annual ryegrass, the impact of N levels on photochemistry of photosystem II (PS II) of NaCl treated annual ryegrass seedlings were firstly assessed through chlorophyll a fluorescence transient-JIP test. The step O to J represents the reduction process of Q_A by PSII. The curve then rise to I phase because of the brimming plastoquinone pool. The step I to P was account for the block of electron transfer to the acceptor side of PSI. According to the results, under control condition, the fluorescence of I and P phase of seedlings leaves grown with 2.0 mM N or 5.0 mM N was stronger than that grown with 0.5 mM N (Fig. 2A). However, when exposed to NaCl, the chlorophyll fluorescence curve of annual ryegrass leaves grown with 2.0 mM N from I to P step was higher than that under 0.5 mM or 5.0 mM N (Fig. 2B, C). Especially, the OJIP curve were much more higher when plants exposed to relatively low NaCl treatment (50 mM) under 2.0mM N level compared to other two N levels (Fig. 2C). The results suggested that nitrogen deficiency or excess under salt stress might lead to the photosynthetic electron transport traffic jam, especially beyond Q_A^-. In addition, under NaCl treatment, the leave chlorophyll content of the plants grown with 2.0 mM and 5.0 mM N was significantly higher compared to that grown with 0.5 mM N. However, there was no significant difference in chlorophyll content between 2.0 mM and 5.0 mM N-supplied plants (Fig. 2D).

Impact of N on Chlorophyll fluorescence parameters in the leaves of ryegrass under NaCl stress

Fluorescence parameters were then used to quantify the photosynthetic behaviour of the samples. Under the control condition, the PI_{ABS} value, which represents the overall activity of PSII, increased with the N level, and achieved a maximum under 5.0 mM N (Fig. 3A). However, under 50 mM or 100 mM NaCl treatment, the PI_{ABS} value under 2.0 mM N were higher than that under other N levels (Fig. 3B, C). The variable fluorescence at J phase (V_j) and the relative speed of Q_A deoxidation (M_0) of NaCl-treated leaves grown with 2.0 mM N were smaller than those grown with 0.5 mM or 5.0 mM N, and the difference was most significant under 50 mM NaCl treatment (Fig. 3B). Under normal condition, Ψ_0 and ΦE_0 displayed no significant difference among three N levels (Fig. 3A). When exposed to 50 mM NaCl, the proportion of energy used for photochemical reaction and energy electron transport in leaves (ϕ_0, ϕE_0) grown with 2.0 mM N were larger than those in the leaves grown with other N levels, together with greater reaction center density RC/CS_0 and electron-transfer energy ET_0/CS_0 and lower DI_0/CS_0 (the energy consumed in unit cross-sectional area) (Fig. 3B). However, ϕP_0, which represents the maximum quantum yield for primary photochemistry, displayed no changes. When exposed to 100 mM NaCl, the PI_{ABS} value under 2.0 mM N were higher than that under other N levels, whereas the other parameters showed no significant change (Fig. 3C). These results suggested that the optimum amount of N might promote primary photochemical reactions of PSII, especially under relatively low NaCl level.

The lipid peroxidation levels and activities of antioxidant enzymes in the leaves of the annual ryegrass seedlings under NaCl stress

Malondialdehyde (MDA) is one of the products of membrane lipid peroxidation which can be used to represent the degree of damage to plants caused by salt. The results showed that there was no significant difference of MDA content among three N levels in the absence of salt stress. When plants grown under 2.0 mM N were exposed to a relative lower NaCl treatment (50 mM), the MDA content showed significantly decrease compared to control. In addition, the MDA content of plants grown under higher N concentration were significantly lower compared to that grown under 0.5 mM N (Fig. 4A). The electrolyte leakage (EL) in the leaves of ryegrass increased with the increase of NaCl concentration under all three N levels. When exposed to 100 mM NaCl, The EL in the leaves of ryegrass grown under higher N concentration was significantly lower compared to that grown under 0.5 mM N. Moreover, under 100 mM NaCl stress, the EL of ryegrass grown under 2.0 mM N was significantly lower than that grown under 5.0 mM N (Fig. 4B). The lipid peroxidation levels and activities of antioxidant enzymes of the leaves were also determined. With the increase of NaCl concentration, the CAT activity presented upward trend under all N levels. Under NaCl treatments, the activity of CAT antioxidant enzyme of ryegrass seedlings cultured at 2.0 mM N was the highest compared with that of plants cultured at 0.5 mM N or 5.0 mM N (Fig. 4C). The activity of POD antioxidant enzyme of ryegrass seedlings showed no obvious regularity with the N levels. However, when exposed to 50 mM NaCl, the POD activity of seedlings grown under 2.0 mM N was higher than

234 seedlings grown under 5.0 mM N (Fig. 4D). These results suggested that ryegrass cultured in 2.0
235 mM N solution might improve the activities of certain antioxidant enzymes and enhance the salt-
236 tolerance ability of ryegrass, especially at relatively low NaCl level.

237 **Effect of different N treatment on the N content and N assimilation-related genes under**
238 **NaCl stress**

239 To investigate the influence of different N treatment on N assimilation under NaCl stress, we
240 checked the expression of NR gene in the leaves of ryegrass, which is a rate limiting enzyme of
241 nitrate assimilation. Without NaCl treatment, the level of NR expression in leaves increased with
242 the increase of N concentration. N reducing (2.0 mM) caused a significant decrease in mRNA
243 expression of NR (Fig. 5A), as compared with 5.0 mM N-applied plants. When plants were
244 cultured with 5.0 mM N, the level of NR gene expression showed a significantly decrease with
245 the increase of salt concentration. However, under 2.0 mM N, the suppression degree of NR
246 expression by salt stress was relatively lower. Compared with 0 mM NaCl, the gene expression
247 of NR of 2.0 mM N-supplied plants showed no significantly decrease when exposed to 100 mM
248 NaCl (Fig. 5A). Under the treatment combined nitrogen and salt, the homolog gene of GS
249 showed a similar expression response pattern with NR (Fig. S2A). The expression of the NRT
250 gene was induced when plants were exposed to a relatively low NaCl level (50 mM). However,
251 there was no significant difference in the homolog of one NRT gene expression between plants
252 grown with 5.0 mM N and 2.0 mM N under NaCl treatment (Fig. S2B). When exposed to NaCl,
253 the nitrogen content of leaves grown under 2.0 mM N or 5.0 mM N showed significantly

decrease compared to that grown under control condition, respectively. Without NaCl treatment, the N content of ryegrass leaves grown under 5.0 mM N was higher. However, under salt treatment, the N content of leaves showed no significant difference between 5.0 mM N and 2.0 mM N application (Fig. 5B).

Discussion

Plant salt tolerance is a complex phenomenon involving morphological, physiological, and biochemical processes. Studies have reported that the application of N may alleviate the toxicities of abiotic stresses in plants (*Correia et al., 2005*; *Siddiqui et al., 2012*; *Singh et al., 2016*). As for salt stress, the mainly harmful on plants are the toxic effects of salt ions, the osmotic effect and nutrient imbalance caused by salt ions (*Kohler et al., 2009*; *Shannon, 1997*). The application of N fertilization has a decisive role on the growth and development of the many plants, and the correct level of N could help to mitigate the damage caused by nutritional imbalances due to saline irrigation (*Al-Rawahy et al., 1992*). However, related research showed that plant growth was significantly affected by interaction between soil salinity and N, but not by N alone (*Papadopoulos & Rendig, 1983*; *Chen et al., 2010*). In this experiment, the exogenous N application significantly increased the plant height and the biomass of the annual ryegrass seedlings and then the nitrogen content, but there was a concentration effect. In the absence of salt, the increment of plant height and biomass increased with the increase of N level, and reached maximum at 5.0 mM N. However, when exposed to NaCl, the plant height and the relative increase of biomass reached maximum at the N level of 2.0 mM (Fig.1). Moreover,

ultra-low N could not promote, but seriously inhibit the growth of ryegrass under both control and salt conditions (Fig. S1). These results were similar with results detected in cotton (*Chen et al., 2010*). Previous study on the annual ryegrass reported that increasing N application could promote shoot growth under salinity of 2.0 and 11.2 dS/m (*Sagi et al., 1997*). However, we noticed that moderate reduction of nitrogen application had the maximum promotion effect on plant growth. This difference may be due to the levels of salt used for treatments. In this study, we are mainly concerned about the optimize nitrogen application at lower salt concentration. Moreover, N content was also positively correlated with the amount of N applied and reached the highest at the N level of 5.0 mM without salt treatment. However, under salt treatments, there was no significant difference in N content of ryegrass leaves between 5.0 mM and 2.0 mM N application (Fig. 5B). External conditions such as salt can stimulate the production of ROS and ROS can further cause damage to lipids in plant cells (*Kohler et al., 2009*). Accumulation of N containing compounds has been reported to participate in salt response such as osmotic adjustment and ROS scavenging (*Dluzniewska et al., 2007*; *Ehlting et al., 2007*; *Homaee et al., 2002*; *Mansour, 2000*; *Song et al., 2006*; *Sudmalis et al., 2018*). In this study, moderately low N application also could reduce the damage to the membrane of ryegrass seedlings caused by salt stress by reducing MDA content or elevating certain antioxidant enzymes activities, especially at relative low NaCl treatment. Together, these results indicated that the saline habitat might change the N requirement of ryegrass seedlings. Excessive or ultra-low N applications both have the opposite effects on the growth or salt resistant of annual ryegrass under low level of salt stress.

Chlorophyll a fluorescence transient is a useful tool to reflect the primary reaction alternations of PSII, which is more sensitive than photosystem I (PS I) in response to salt stress. To investigate PSII behaviors in O-J-I-P transient, JIP test is always used to quantify the derived photochemical parameters (*Apostolova et al., 2006*; *Sayed, 2003*; *Stirbet et al., 2014*). In this study, when the annual ryegrass plants were exposed to NaCl, the nitrogen application level had a significant effect on fluorescent transients, especially the J and P steps (Fig. 1). N deficiency and N over application under salt stress might lead to the photosynthetic electron transport traffic jam, especially beyond Q_A^- (Fig. 2B, C). With the increase of N level, the parameter of PI_{ABS}, which could accurately reflect the state of plant photosynthetic apparatus, showed an upward trend without NaCl treatment (Fig. 2A), indicating that N could promote the primary photochemical reactions of PSII in the waterside. However, in the saline habitat, if the N is excessive or deficiency, the promotion of the primary photochemical reaction of the PSII will be slow or even reduced (Fig. 2B, C). In addition, the accumulated amount of Q_A^- (V_j) and the relative speed of Q_A deoxidation (M_0) (*Strasser, 1997*; *Strasserf & Srivastava*, 1995; *Force et al., 2003*) of plants grown under moderately low N were smaller than those grown under other N conditions, indicating that leaves grown under moderate N level have a higher electron transport rate between Q_A and Q_B, thus reducing the accumulation amount of Q_A^- and increasing the photochemical reaction efficiency (Allakhverdiev & Murata, 2004). The increase of Ψ_0 and ΦE_0 of plants grown under 2.0 mM N indicated that leaves use more energy for photochemical reaction and electron-transfer process, thus producing more NADPH for carbon assimilation and proving that leaves have the optimal energy distribution under certain salt level (*Strasser et al.,*

316 *2004*). The leaves of annual ryegrass grown under 2.0 mM N also have a greater reaction center
317 density RC/CS_0 and higher ET_0/CS_0 but lower DI_0/CS_0 than those grown under other N
318 conditions. This pattern indicated that the specific activity of a unit cross-sectional area of leaves
319 grown under moderately low N was stronger than that grown under other N conditions, reducing
320 the energy burden of a unit reaction center. We also noticed that, under the lowest NaCl
321 treatment (50 mM), the application of 2.0 mM N had the best effect on alleviating salt stress.
322 Under 50 mM NaCl treatment, the physiological indexes of annual ryegrass seedlings seemed
323 less affected, and therefore it might be more sensitive to the promotion of nitrogen application.
324 Thus, we proposed that the optimum amount of N might promote primary photochemical
325 reactions of PSII under certain level of NaCl treatment.

326 After absorption with ammonia N, it can be directly assimilated by plants. After absorption
327 of nitrate N, it must first be reduced by nitrate reductase and sub-acid reductase. NR can reduce
328 nitrate N to ammonium, and it also has important effects on photosynthesis and other processes
329 of N metabolism (*Xu et al., 2012*). Reports have shown that NO_3^- has a significant effect on the
330 induction of NR expression. From the results of this experiment, the level of NR gene expression
331 in leaves increased with the increase of N concentration under control condition (Fig. 5A, B),
332 which is consistence with the previous reports (*Oaks, 1993*). However, if the seedlings were
333 treated with NaCl, the NR expression level was significantly decreased at higher N level (5.0
334 mM). On the contrary, at a moderately low N level (2.0 mM), the NR expression level is
335 relatively low without NaCl treatment, but the degree of reduction is moderate when exposed to
336 NaCl. The GS gene expression showed a similar trend with the NR gene under the interaction

between salt and nitrogen conditions, indicating a cooperative response mechanism between N assimilation-related genes (Fig. S2A). Therefore, it can be seen that moderate N application might help annual ryegrass maintain the expression level of N assimilation-related gene (Fig. 5A) and further maintain the nitrogen content under salt stress (Fig. 5B). However, when excessive N was applied under salt stress, the *NR* expression was significantly increased, indicating that N assimilation was strengthened; it might then compete with photosynthetic carbon to compete for the assimilation forces produced by photosynthesis photoreaction, namely ATP and NADPH and increase the burden of photosynthetic electron transfer. The competition result might lead to a decrease of the overall activity of PSII of annual ryegrass (PI_{ABS}) (Fig. 2). Under nitrogen deficiency, the reduced absorption of nitrogen might reduce the consumption of nitrogen assimilation reducing power, most of which are derived from photosynthesis, thus resulting in the accumulation of chloroplast NADPH. The over-accumulation of NADPH could inhibit the photosynthetic efficiency and cause excessive production of ROS (Fig. 3A), leading to increased cell membrane damage, which may in turn lead to reduced photosynthetic efficiency (Fig. 2B, C, D). Nitrogen is also one component of chlorophyll which is not only the most important pigment molecules of photosynthesis involved in energy absorption and transmission but also the essential electron mediator during electron transport. Studies showed that the nitrogen content of leaf is constant with the photosynthetic capacity (*Grassi et al., 2005*; *Kattge et al., 2009*). Through this experiment, we can see that under different salt stress condition, the appropriate addition of N can indeed increase the relative content of chlorophyll in plants. However, the relative content of chlorophyll is only positively correlated with N levels within a certain range

(0.5-2.0 mM) and should be reduced beyond a certain range (Fig. 2D). The moderately supply of N under salt stress increased the content of chlorophyll and might increase the light-harvesting ability, partly contributing to the up-regulated photosynthetic performance index. Based on the above studies, it can be seen that moderately low N application under low level of salt stress might help annual ryegrass maintain the expression level of N assimilation-related gene and then maintain the leaf N content of the plant, which might in turn changes the chlorophyll, further avoiding the negative effect on photosynthetic capacity.

Conclusion

To investigate the possible mechanism of moderately low nitrogen-mediated alleviation of NaCl stress, the degree of lipid peroxidation, antioxidant enzyme activity alternation, changes of photosynthesis performance and nitrogen assimilation were analyzed in this study. In summary, under low level of salt stress, the demand for N may have decreased and moderately reducing N application could help to alleviate the damage caused by salt stress in annual ryegrass mainly by alleviating the damage caused by ROS and promoting the performance of photosynthesis and nitrogen metabolism. Further, in order to enhance plant growth and increase nitrogen use efficiency, the optimum application of nitrogen fertilizer needs to be controlled to match the plant needs at each growth stage and to adapt to different salt environment.

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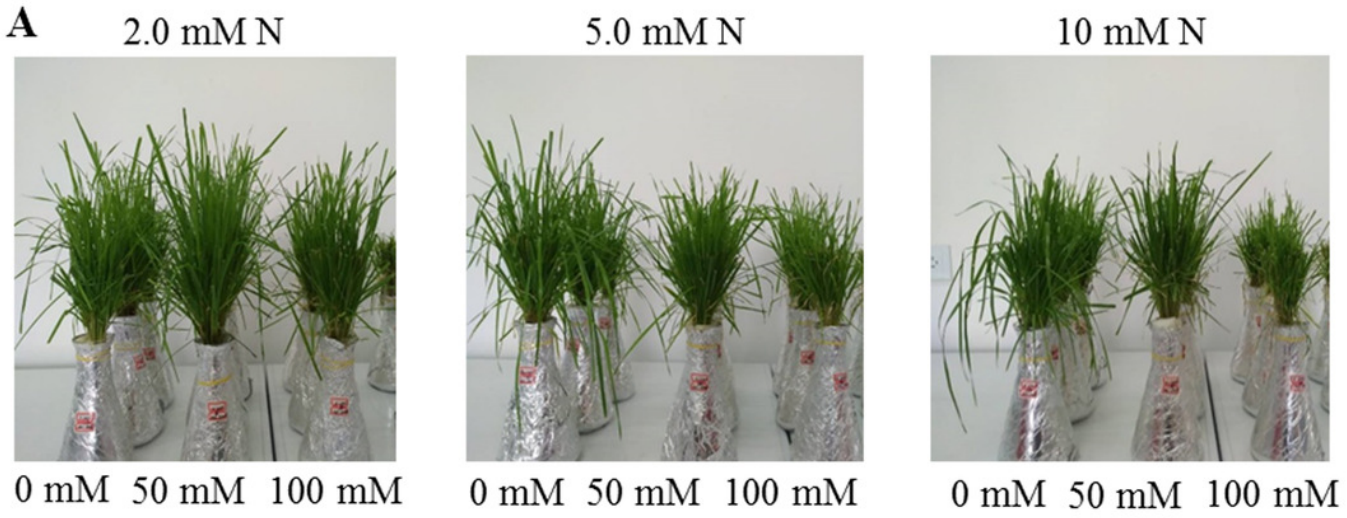
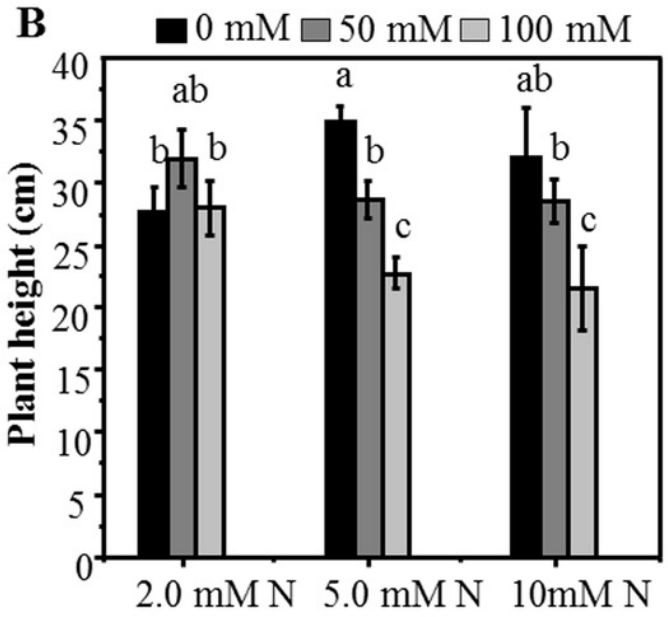
Figure 1

Morphological parameters of annual ryegrass seedlings grown under different nitrogen and salt conditions.

Fig. 1. Morphological parameters of annual ryegrass seedlings grown under different nitrogen and salt conditions. The seeds of annual ryegrass were cultured in soil for one month, and the seedlings cut to the same height were then transferred into different nitrogen level (2.0, 5.0, 10 mM) under NaCl (0, 50, 100 mM) stress in a hydroponic culture. After being grown for 10 days, the plant height and biomass were measured. (A) Images of seedlings at 10 days after transferred. (B) Plant height at 10 days after transferred. (C) The relative change of biomass (% of biomass which was measured before treatment). Different letters above the columns indicate significant differences at $P < 0.05$ by Student's *t*–test.


A
2.0 mM N
5.0 mM N
10 mM N
0 mM 50 mM 100 mM
0 mM 50 mM 100 mM
0 mM 50 mM 100 mM


B
0 mM 50 mM 100 mM
Plant height (cm)
40
35
30
25
20
15
10
5
0
ab
b
b
a
b
c
ab
b
c
2.0 mM N
5.0 mM N
10mM N

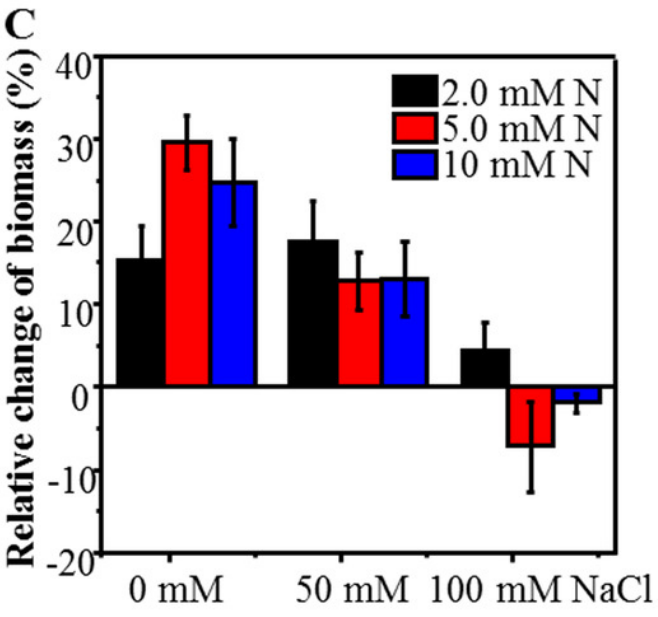

C
Relative change of biomass (%)
40
30
20
10
0
-10
-20
2.0 mM N
5.0 mM N
10 mM N
0 mM
50 mM
100 mM NaCl

Figure 2

Alterations of chlorophyll fluorescence transients in leaves of annual ryegrass.

The annual ryegrass were grown with different nitrogen concentrations (0.5, 2.0, 5.0 mM) under 0 mM, 50 mM (B), 100 mM NaCl (C) stress respectively. (D) Influence of nitrogen concentration on chlorophyll content under different levels of NaCl stress respectively. Different letters above the columns indicate statistically significant differences at $P < 0.05$ by Student's *t*–test.

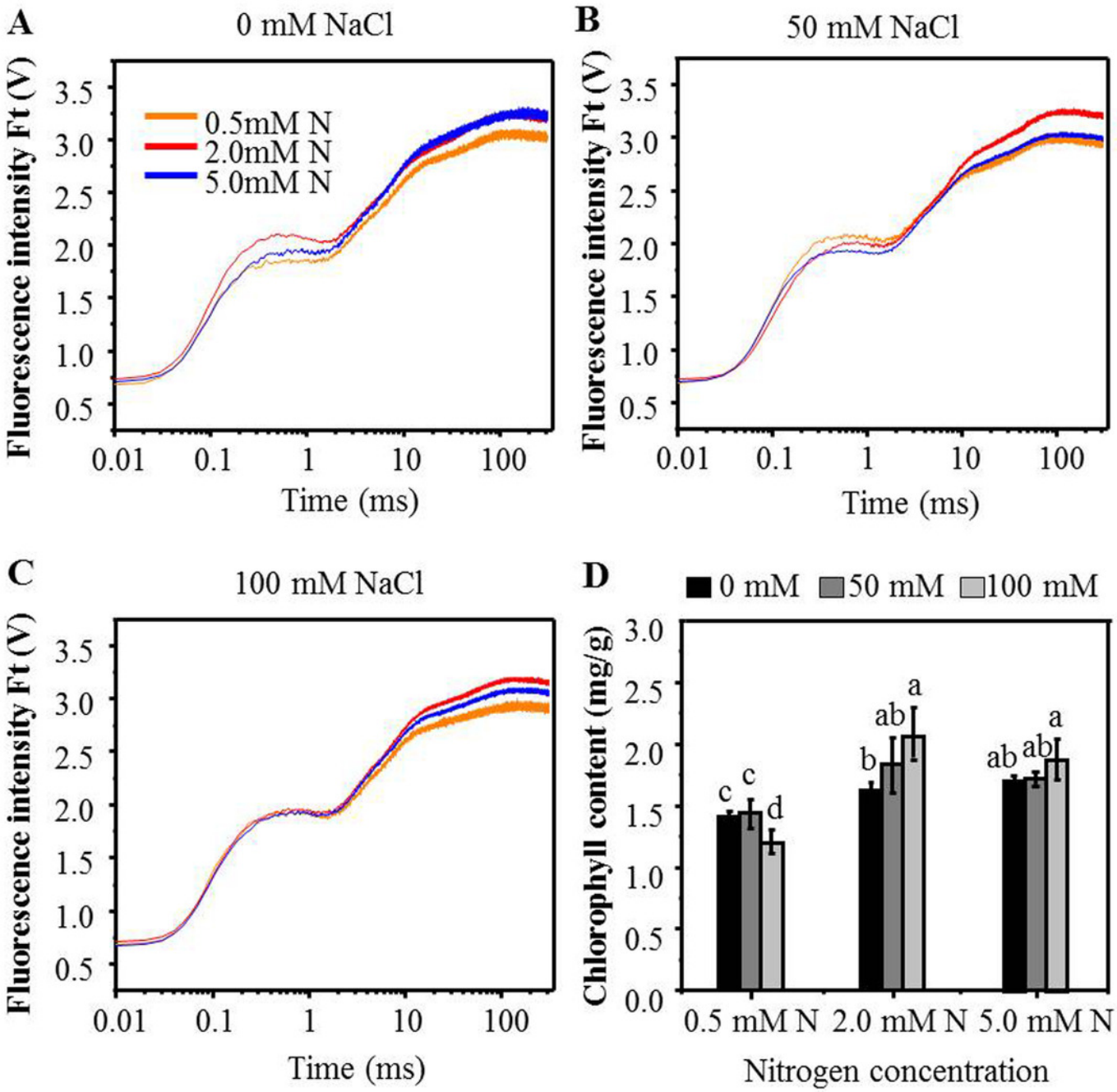

A
0 mM NaCl
0.5mM N
2.0mM N
5.0mM N
Fluorescence intensity Ft (V)
Time (ms)
B
50 mM NaCl
Fluorescence intensity Ft (V)
Time (ms)
C
100 mM NaCl
Fluorescence intensity Ft (V)
Time (ms)
D
0 mM
50 mM
100 mM
Chlorophyll content (mg/g)
0.5 mM N
2.0 mM N
5.0 mM N
Nitrogen concentration

Figure 3

“Radar plots” of picked parameters characterizing influence of nitrogen concentration (0.5, 2.0, 5.0 mM N) on PS II of annual ryegrass.

The annual ryegrass leaves exposed to 0 mM (A), 50 mM (B), 100 mM (C) NaCl stress respectively. All values are shown as percent of control. The parameters of plants grown under 0.5 mM nitrogen concentration were set as control. Control=1. *indicate parameters statistically significant between different N levels under the same NaCl level.

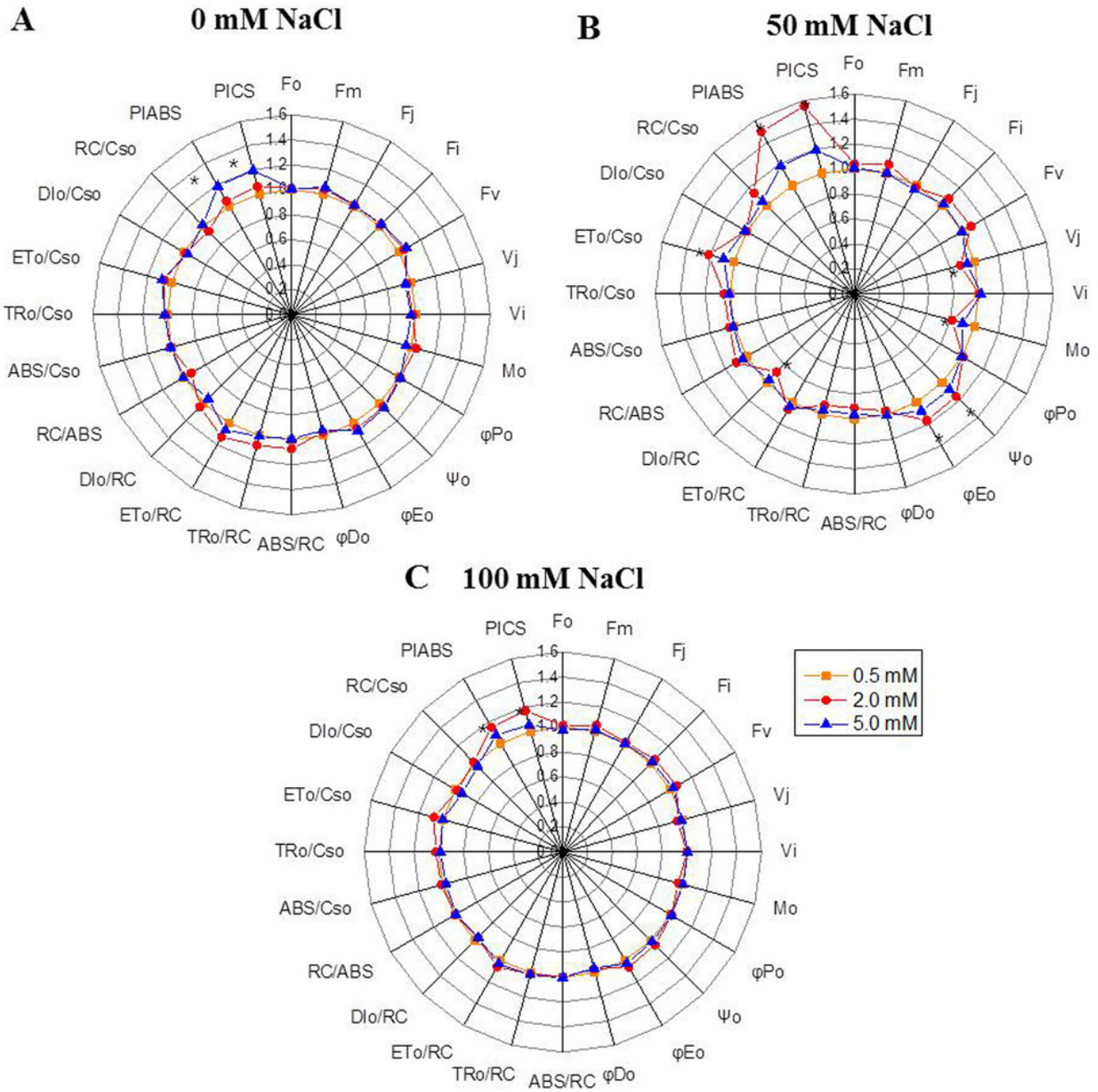

A
0 mM NaCl
B
50 mM NaCl
C
100 mM NaCl
PICS
Fo
Fm
Fj
Fi
Fv
Vj
Vi
Mo
φPo
Ψo
φEo
φDo
ABS/RC
TRo/RC
ETo/RC
DIo/RC
RC/ABS
ABS/Cso
TRo/Cso
ETo/Cso
DIo/Cso
RC/Cso
PIABS
0.5 mM
2.0 mM
5.0 mM

Figure 4

The membrane damage degree and antioxidant enzymes activities of the annual ryegrass leaves.

MDA content (A), EL (B), catalase (CAT) (C) or peroxidase (POD) (D) activity in the leaves of annual ryegrass grown with different nitrogen concentrations (0.5, 2.0, 5.0 mM N) exposed to different NaCl level (0, 50, 100 mM NaCl) respectively. Different letters above the columns indicate statistically significant differences at $P < 0.05$ by Student's t–test.

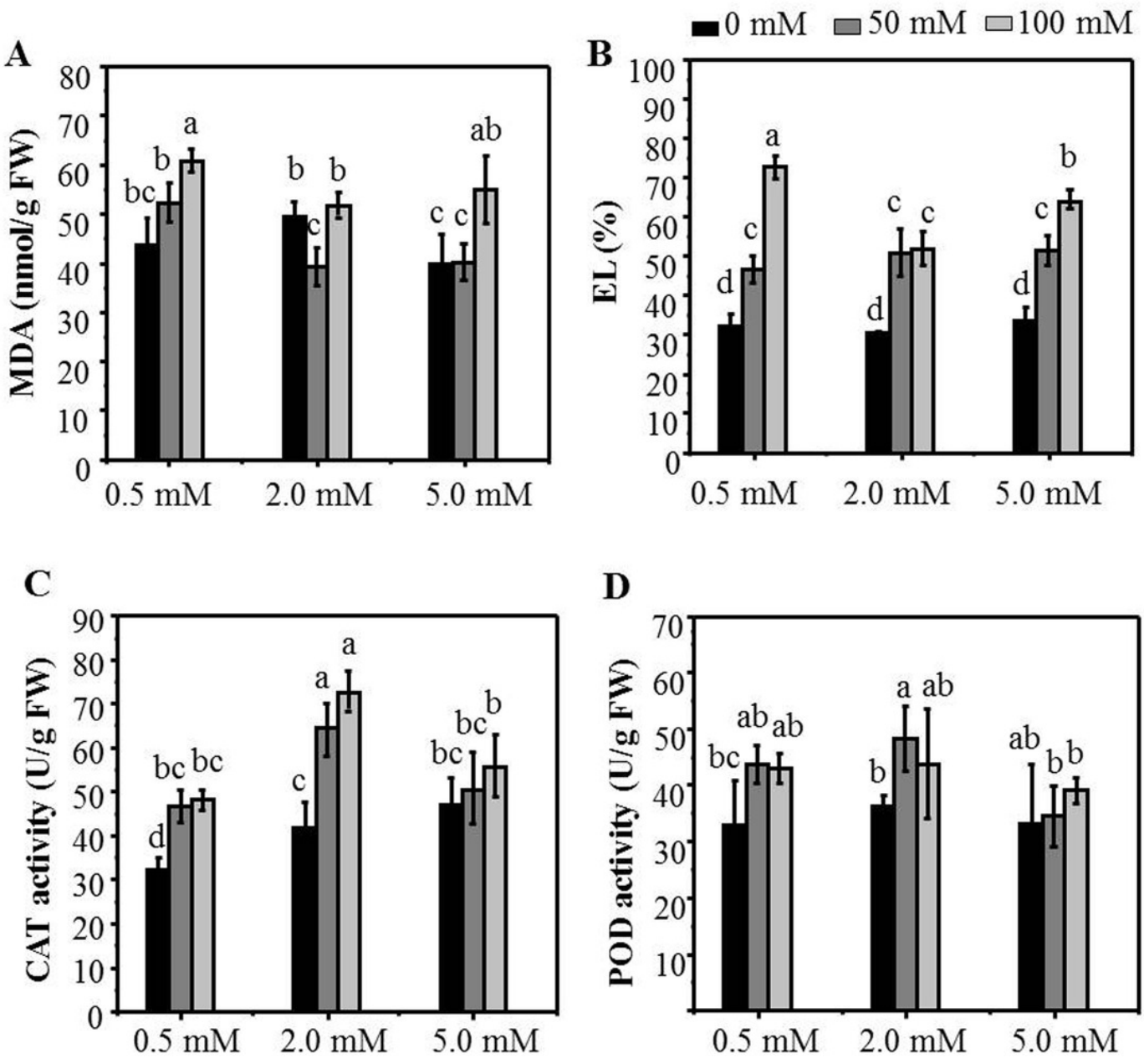

A
MDA (nmol/g FW)
0 mM
50 mM
100 mM
B
EL (%)
C
CAT activity (U/g FW)
D
POD activity (U/g FW)
0.5 mM
2.0 mM
5.0 mM

Figure 5

Relative expression of N metabolism-related genes and nitrogen content of leaves grown under different conditions.

(A) NR expression in the leaves of annual ryegrass grown under different nitrogen concentration (2.0, 5.0 mM) exposed to different salt stress for 12 hours (0, 50, 100 mM NaCl) respectively; (B) Nitrogen content of leaves grown with different nitrogen concentrations exposed to different salt stress for 10 days respectively. Different letters above the columns indicate statistically significant differences at $P < 0.05$ by Student's *t*–test.