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 salt stress-induced damage on plants. This study explores the changes of nitrogen requirement in feeding annual ryegrass seedlings under mild salt concentrations (50 mM, 100 mM) plus its underlying mitigation mechanism. Results showed that low salt concentration decreased N requirement as observed from the increment in plant height and biomass at a relative low N level (2.0 mM not 5.0 mM). Under salt treatment, especially at 50 mM NaCl, the OJIP (Chl a fluorescence induction transient) curve and a series of performance indexes (PI_{ABS}, RC/CS_0, ET_0/CS_0, φE_0, φ_0) peaked 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 exhibited 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 mild salt treatment of annual ryegrass seedlings might reduce N requirement while moderately low N application could promote their growth via regulating photosynthesis, alleviating ROS-induced (reactive oxygen species) damage and maintenance of N metabolism. 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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2 **ryegrass seedlings**

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17   **Abstract**

18   Appropriate application of nitrogen (N) can alleviate the salt stress-induced damage on plants.

19   This study explores the changes of nitrogen requirement in feeding annual ryegrass seedlings

20   under mild salt concentrations (50 mM, 100 mM) plus its underlying mitigation mechanism.

21   Results showed that low salt concentration decreased N requirement as observed from the

22   increment in plant height and biomass at a relative low N level (2.0 mM not 5.0 mM). Under salt

23   treatment, especially at 50 mM NaCl, the OJIP (Chl a fluorescence induction transient) curve and

24   a series of performance indexes (PI_{ABS}, RC/CS_0, ET_0/CS_0, ϕE_0, ϕ_0) peaked whereas DI_0/RC, V_j

25   and M_0 were the lowest under moderately low N level (2.0 mM). In addition, under salt stress,

26   moderately low N application could maintain the expression of NR (nitrate reductase) and GS

27   (glutamine synthetase) encoding genes at a relatively stable level but had no effect on the

28   expression of detected NRT (nitrate transporter) gene. The seedlings cultured at 2.0 mM N also

29   exhibited the highest activity of CAT and POD antioxidant enzymes and the lowest MDA

30   content and EL under relative low level of salt treatment. These results indicated that mild salt

31   treatment of annual ryegrass seedlings might reduce N requirement while moderately low N

32   application could promote their growth via regulating photosynthesis, alleviating ROS-induced

33   (reactive oxygen species) damage and maintenance of N metabolism. These results also can

34   provide useful reference for nitrogen application in moderation rather than in excess on annual

35   ryegrass in mild or medium salinity areas through understanding the underlying response

36   mechanisms.

Introduction

Feeding annual ryegrass (*Lolium multiflorum* Lam.) is an important forage grass with high

yield, good palatability and high nutritive value (*Castanheira et al., 2014*). Salt stress is one of

the major abiotic factors that limit annual ryegrass growth and productivity. The adverse effects

of salt stress on plants include ionic toxicity, osmotic stress and secondary stresses, such as a

decline in photosynthesis, oxidative stress and nutritional disorders (*Allakhverdiev & Murata,*

2004; *Kalaji et al., 2011*; *Zhu, 2001*). The forage quality such as crude protein, organic matter

can be adversely affected by elevated salinity (*Ibrahim et al., 2018; Robinson et al., 2004*). In

response, through long term evolution, plants have adopted a series of response mechanisms to

resist and minimize salt stress-induced damage (*Deinlein et al., 2014*; *Zhao et al., 2020*). For

example, in response to salt-induced oxidative stress, a series of antioxidant enzymes were

induced to scavenge excessively produced reactive oxygen species (ROS) (*Kohler et al., 2009*;

Bady et al., 2010), such as superoxide dismutase (SOD), peroxidase (POD), and catalase (CAT)

(*Apel & Hirt, 2004*; *Dong et al., 2001*).

In saline environment, nitrogen (N) is usually the limiting growth macro-nutrient, thus N

application is the most effective method of improving plant growth under salt stress. The mainly

available inorganic N forms are ammonium N (NH_4^+) and nitrate N (NO_3^-), which are

transported by AMT (Ammonium transporter) and NRT (Nitrate transporter) respectively

(*Giagnoni et al., 2015*). The inorganic N is then assimilated and converted into amino acid via

57   several enzymes such as nitrate reductase (NR), glutamine synthetase (GS) and glutamine 2-

58   oxoglutarate aminotransferase (GOGAT) (*Xu et al., 2012*). Salt stress has also proved to inhibit

59   the activity of many enzymes such as NR and GS/GOGAT, involved in N assimilation in maize

60   (*Zea mays*), cowpea (*Vigna unguiculata*) mung bean (*Vigna radiata*), tomato (*Solanum*

61   *lycopersicum*) and rice (*Oryza sativa*) (*Chakrabarti & Mukherji, 2007*; *Debouba et al., 2007*;

62   *Khan & Srivastava, 1998*; *Parul et al., 2015*; *Silveira et al., 2001*; *Wang et al., 2012*), and then

63   decreased the absorption and utilization of N in plants (*Singh et al., 2016*). Increased salinity was

64   also reported to significantly reduce nitrogen use efficiency (*Murtaza et al., 2013*). On the

65   contrary, some studies showed that processes related to N uptake and assimilation in some plant

66   species were stimulated under certain levels of salt stress. For instance, salt could induce the

67   expression level of nitrate transporter genes such as *McNRT1* (*Popova et al., 2003*). Under salt

68   exposure, the nitrate uptake rate and activity of NR were promoted in glasswort (*Salicornia*

69   *europaea*) (*Nie et al., 2015*). Thus, it has been speculated that the alteration of plant N nutrition

70   level may hold great promise for regulating salinity response in different species under certain

71   salt level (*Chen et al., 2014*).

72   Generally, N application can ameliorate the negative effects of salinity by compensating

73   and correcting nutritional imbalances in higher plants (*Esmaili et al., 2008*; *Gómez et al., 1996*;

74   *Mansour, 2000*; *Villa et al., 2003*). Several N-containing compounds were accumulated in plants

75   that were subjected to salt stress (*Dluzniewska et al., 2007*; *Ehlting et al., 2007*; *Sudmalis et al.,*

76   *2018*). The accumulates were then reported to participate in osmotic adjustment, promotion of

77 photosynthetic capacity and mitigation of oxidative stress by scavenging excessive ROS

78 (*Homaee et al., 2002*; *Mansour, 2000*; *Rontein et al., 2002*; *Song et al., 2006; Todorova et al.,*

79 *2013*). Although many studies have shown that N plays an important role in the amelioration of

80 salt stress, it has been confirmed that the alleviation of salt inhibition from N application shows

81 concentration effects. For example, at the higher salinity levels, increasing the N content was

82 found to be ineffective in resisting negative influences caused by the enhanced salt

83 concentrations in tomato (*Papadopoulos & Rendig, 1983*). Another previous study reported that

84 low levels of N could mitigate the negative effects whereas high N levels may exacerbate the

85 detrimental effects of salt stress on photosynthetic rate of chickpea leaves (*Soussi et al., 1998*) .

86 A recent study also found that, low to moderate N application could mitigate the adverse effects,

87 while excessive N could elevate the negative effects of salt stress on cotton growth (*Chen et al.,*

88 *2010*). In maize, reduction in N application could not cause additional damage on plant

89 development under salt stress based on reductions in evapotranspiration *(Lacerda et al., 2016).*

90 Other studies also pointed out that excessive nitrogen fertilization under high salinity levels

91 might lead to more pronounced osmotic effect which in turn provokes the negative effect on crop

92 yield (*Beltrão et al., 2002*). In addition, in highly saline soils, excessive application of N

93 fertilizer will trigger secondary soil salinization, which in turn adversely affects crop growth

94 *(Chen et al., 2010*). Moreover, over fertilization with N may contribute to N leaching in the

95 saline soil, where plants are unable to utilize the supplied N fertilizer efficiently and leading to

96 groundwater pollution (*Pessarakli & Tucker, 1998*; *Shenker et al., 2003*, *Ward, 2013*).

97   Therefore, N requirements for plants under saline environment might be inconsistent with

98   those in normal environment. The difference might be attributed to distinct physical and

99   chemical properties of growth medium and the alteration of plants nitrogen use efficiency plus

100   other physiological response. Hence, in order to minimize the negative effects of salinity as well

101   pollution of underground water, proper N fertilizer management in plants is necessary for

102   different salt conditions. A previous study on annual ryegrass reported that increasing N

103   concentration in the nutrient solution enhanced shoot biomass production under relatively high

104   salinity levels (*Sagi et al., 1997*). However, at relatively low salt concentrations, the optimization

105   of nitrogen demand and the possible mechanisms underlying this alleviation in annual ryegrass

106   are still not fully explored. Based on the above studies, the objectives of this work were to assess

107   the optimal N level under relatively low salt level and to investigate the possible salt stress-

108   alleviatory mechanisms by analyzing physiological indexes and metabolism of N in annual

109   ryegrass seedlings.

110   **Materials and Methods**

111   **Plant materials and growth conditions**

112   Annual ryegrass seeds were thawed in plastic pots that were filled with plant growth medium and

113   then cultured in the greenhouse under natural sunlight. After one month, the seedlings were then

114   transferred into Erlenmeyer flasks containing 585mL of nutrient solution. The seedlings were

115   then mowed to a height of 12.5 cm before the treatments were initiated. The experiment was set

116   as control (0 mM NaCl) and NaCl treatment (50 mM or 100 mM). Both the control and NaCl

117 regimes included different nitrogen application level (using NH_4NO_3 as nitrogen source). The

118 hydroponic culture media was processed in a growth chamber under the following conditions:

119 22/18°C (day/night), 65% relative humidity, 300 μmol $m^{-2}s^{-1}$ photons and a 16-h day/8-h night

120 cycle. The culture solution was refreshed every two days.

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

122 A pulse amplitude modulation fluorometer (PAM2500, Heinz Walz GmbH) was used to measure

123 the chlorophyll a fluorescence transient. In brief, the annual ryegrass leaves were put in dark

124 place for 30 min, and the leaves were then exposed to 3,000 μmol photons m^{-2} s^{-1} red light

125 condition. Each treatment consisted of at least three replicates. Based on the theory of energy

126 fluxes in biofilm, the JIP test can further translate the primary data into other biophysical

127 parameters (*Force et al., 2003*). Hence, the basic parameters were used to calculate a series of

128 parameters (*Yusuf et al., 2010*).

129 **Chlorophyll content and electrolyte leakage**

130 The SPAD 502 Plus Chlorophyll Meter (SPAD-502Plus, Spectrum Technologies, Inc., USA)

131 was used to quantify the leaf chlorophyll content. The electrolyte leakage (EL) was measured

132 according to the previous method (*Blum & Ebercon, 1981*).

133 **Enzymes activity and lipid peroxidation**

134 Fully expanded of 0.3 g leaves were immediately grounded into powder with liquid N. 4 mL ice-

135 cold phosphate buffer (50 mM, pH 7.8) was added into the powder and the samples were

136   centrifuged at 12,000 rpm for 20 min at 4℃. The supernatant was collected to measure the

137   activity of POD and CAT and the content of MDA based on the method as described by previous

138   study (*Fu & Huang, 2001*).

139   **Quantitative RT-PCR Analysis**

140   The total RNA was isolated and reverse transcribed using the RNeasy kit (Qiagen) and TaqMan

141   reverse transcription kit (Applied Biosystems). Quantitative real-time RT-PCR analysis was

142   conducted using SYBR Green real-time PCR master mix (Toyobo, Japan) and ABI real-time

143   PCR system (Applied Biosystems, FosterCity, CA). The primers used are listed in Supplemental

144   Table S1. The ryegrass *Actin* gene was used as an inner control, and comparative Ct method was

145   applied for gene expression level analysis.

146   **Statistical Analysis**

147   One-way ANOVA was performed using SPSS17.0 for Windows (SPSS). All of above tests had

148   at least three independent replicates. Results were expressed as mean ± *SD*, and letters show

149   significant differences *(P < 0.05)* by Student's *t*–test..

150   **Results**

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

152   **stress**

153   Under control condition, the plant height and the relative increase of biomass of annual ryegrass

154   seedlings peaked under 5.0 mM N, and then slightly declined under 10 mM N. As compared to

155   5.0 mM N, the height of seedlings grown under 2.0 mM N and 10 mM N declined by about 10%

156   and 15% respectively (Fig. 1A, B). However, under 50 mM or 100 mM NaCl treatment, the plant

157   height reached maximum under 2.0 mM N and then declined slightly under 5.0 mM N. In

158   addition, salt treatment dramatically reduced the height of plants grown under 5.0 mM or 10 mM

159   N compared with their respective control plants. However, under 2.0 mM N condition, the plant

160   height had no significant difference with or without salt treatment (Fig. 1B). When exposed to 50

161   mM NaCl, the relative increase of biomass had no significant difference among different N

162   levels. After the plants were exposed to 100 mM NaCl for 10 days, the biomass only increased

163   when 2.0mM N was applied whereas the biomass decreased when extra nitrogen (5.0 or 10 mM

164   N) was applied (Fig. 1C). These similar alteration trends in biomass and plant height suggested

165   that salt stress might change the N requirement of annual ryegrass and moderately reducing N

166   application might alleviate the inhibitory effect of salt stress on annual ryegrass seedlings growth.

167   In order to investigate whether ultra-low N treatment still had a moderating function on the

168   growth of annual ryegrass seedlings under salt stress, we added a lower concentration (0.5 mM)

169   of N treatment and conducted another experiment. The results showed that the plant height of

170   annual ryegrass seedlings achieved maximum under 5.0 mM N without salt treatment. However,

171   when exposed to 50 mM or 100 mM NaCl, the plant height reached maximum under 2.0 mM N

172   (Fig. S1A, B), showing the similar tendency with the first experiment (Fig. 1A, B). In addition,

173   without NaCl treatment, there was no significant difference of plant height when seedlings were

174   supplemented with lower N concentration (0.5 mM or 2.0 mM). However, under NaCl treatment,

175   the plant height was significantly increased under 2.0 mM N compared with 0.5 mM N (Fig. S1).

176   The results of these two experiments at different times together confirmed that the alleviatory

177   effect of N application on the growth inhibition of annual ryegrass under salt stress might have a

178   certain range. Moderately low N could alleviate the inhibition of annual ryegrass growth by salt

179   stress through a series of response mechanisms, whereas ultra-low N could not promote, but

180   seriously inhibit the growth of annual ryegrass.

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

182   In order to decipher the effect of moderate N-mediated alleviation of salt stress on annual

183   ryegrass, the impact of N levels on photochemistry of photosystem II (PS II) of NaCl treated

184   annual ryegrass seedlings were determined through chlorophyll a fluorescence transient-JIP test.

185   The step O to J represents the reduction process of Q_A by PSII. Due to the brimming of the

186   plastoquinone pool, the curve then rose to I phase. The step I to P was an account for the

187   blockage of electron transfer to the acceptor side of PSI. Under control condition, the

188   fluorescence of I and P phase of seedlings leaves grown with 2.0 mM N or 5.0 mM N was

189   stronger than that grown with 0.5 mM N (Fig. 2A). However, after NaCl exposure, the

190   chlorophyll fluorescence curve of annual ryegrass leaves grown with 2.0 mM N from I to P step

191   was higher than that under 0.5 mM or 5.0 mM N (Fig. 2B, C). In particular, the OJIP curve was

192   much higher when plants exposed to relatively low NaCl treatment (50 mM) under 2.0mM N

193   level compared to other two N levels (Fig. 2C). The results suggested that deficient or excessive

194   nitrogen application under salt stress might lead to the photosynthetic electron transport traffic

195   jam, especially beyond Q_A^-. In addition, under NaCl treatment, the leave chlorophyll content of

196   the plants grown with 2.0 mM and 5.0 mM N was significantly higher than that grown with 0.5

197   mM N. However, there was no significant difference in the chlorophyll content between 2.0 mM

198   and 5.0 mM N-supplied plants (Fig. 2D).

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

200   **stress**

201   The chlorophyll fluorescence parameters were used to quantify the photosynthetic behavior of

202   the samples. Under the control condition, the PI_{ABS} value, which represents the overall activity of

203   PSII, increased with the N level, and peaked under 5.0 mM N (Fig. 3A). However, under 50 mM

204   or 100 mM NaCl treatment, the PI_{ABS} value under 2.0 mM N were higher than that under other N

205   levels (Fig. 3B, C). The variable fluorescence at J phase (V_j) and the relative speed of Q_A

206   deoxidation (M_0) of NaCl-treated leaves grown with 2.0 mM N were smaller than those grown

207   with 0.5 mM or 5.0 mM N, and the difference was most significant under 50 mM NaCl treatment

208   (Fig. 3B). Under normal condition, Ψ_0 and ΦE_0 had no significant difference among three N

209   levels (Fig. 3A). When exposed to 50 mM NaCl, the proportion of energy used for

210   photochemical reaction and energy electron transport in leaves (ϕ_0, ϕE_0) grown with 2.0 mM N

211   were larger than those grown with other N levels, together with greater reaction center density

212   RC/CS_0 and electron-transfer energy ET_0/CS_0 and lower DI_0/CS_0 (the energy consumed in unit

213   cross-sectional area) (Fig. 3B). However, ϕP_0, which represents the maximum quantum yield for

214   primary photochemistry, displayed no changes. When exposed to 100 mM NaCl, the PI_{ABS} value

215 under 2.0 mM N was higher than that under other N levels, whereas the other parameters showed

216 no significant change (Fig. 3C). These results suggested that the optimum amount of N might

217 promote primary photochemical reactions of PSII, especially under relatively low NaCl level.

218 **The lipid peroxidation levels and activities of antioxidant enzymes in the leaves of the**

219 **annual ryegrass seedlings under NaCl stress**

220 Malondialdehyde (MDA) is one of the products of membrane lipid peroxidation which can be

221 used to assess the degree of the salt-induced damage to plants. The results showed that, in the

222 absence of salt stress, there was no significant difference of MDA content among the three N

223 levels. When the plants grown under 2.0 mM N were exposed to a relatively lower NaCl

224 treatment (50 mM), the MDA content declined significantly compared to the control. In addition,

225 the MDA content of plants grown under higher N concentration were significantly lower

226 compared to those grown under 0.5 mM N (Fig. 4A). The electrolyte leakage (EL) in the leaves

227 of ryegrass increased with the increase of NaCl concentration under all three N levels. When

228 exposed to 100 mM NaCl, The EL in the leaves of ryegrass grown under higher N concentration

229 was significantly lower compared to that grown under 0.5 mM N. Moreover, under 100 mM

230 NaCl stress, the EL of ryegrass grown under 2.0 mM N was significantly lower than that grown

231 under 5.0 mM N (Fig. 4B). The lipid peroxidation levels and activities of antioxidant enzymes of

232 the leaves were also determined. With the increase of NaCl concentration, the CAT enzyme

233 activity rose under all N levels. Under NaCl treatments, the CAT activity of ryegrass seedlings

234 cultured at 2.0 mM N was the highest compared with that of plants cultured at 0.5 mM N or 5.0

235   mM N (Fig. 4C). The POD enzyme activity had no obvious regularity with the N levels.

236   However, when exposed to 50 mM NaCl, the POD activity of seedlings grown under 2.0 mM N

237   was higher than seedlings grown under 5.0 mM N (Fig. 4D). These results suggested that

238   ryegrass cultured in 2.0 mM N solution might improve the activities of certain antioxidant

239   enzymes and enhance the salt-tolerance ability of ryegrass, especially at relatively low NaCl

240   level.

241   **Effect of different N treatment on the N content and N assimilation-related genes under**

242   **NaCl stress**

243   To investigate the influence of different N concentrations on N assimilation under NaCl

244   stress, we assessed the expression level of *NR* gene in the leaves of ryegrass. Without NaCl

245   treatment, the level of the *NR* expression in the leaves increased with the increase of N

246   concentration. A reduction in N (2.0 mM) caused a significant decline in mRNA expression of

247   *NR* (Fig. 5A), as compared with 5.0 mM N-applied plants. When plants were cultured with 5.0

248   mM N, the level of *NR* gene expression showed a significantly decrease with the increase of salt

249   concentration. However, under 2.0 mM N, the suppression degree of *NR* expression by salt stress

250   was relatively lower. Compared with 0 mM NaCl, the expression level of *NR* of 2.0 mM N-

251   supplied plants showed no significant decrease when exposed to 100 mM NaCl (Fig. 5A). Under

252   the combined treatment of nitrogen and salt, the homolog gene of *GS* showed a similar

253   expression response pattern with *NR* (Fig. S2A). The expression of the *NRT* gene was induced

254   when plants were exposed to a relatively low NaCl level (50 mM). However, there was no

255 significant difference in the homolog of one *NRT* gene expression between plants grown with 5.0

256 mM N and 2.0 mM N under NaCl treatment (Fig. S2B). When exposed to NaCl, the nitrogen

257 content of leaves grown under 2.0 mM N or 5.0 mM N showed a significant decline compared to

258 the control condition, respectively. Without NaCl treatment, the N content of ryegrass leaves

259 grown under 5.0 mM N was higher. However, under salt treatment, the N content of leaves

260 showed no significant difference between 5.0 mM N and 2.0 mM N application (Fig. 5B).

261 **Discussion**

262   Plant response to salt stress is a complex phenomenon that involves both morphological,

263 physiological, and biochemical processes. It has been reported that the application of N may

264 alleviate salt stress-induced phytotoxicity (*Correia et al., 2005*; *Siddiqui et al., 2012*; *Singh et al.,*

265 *2016*). Salt stress effect on plants consists of Na^+ and Chl^- ion toxicity, osmotic effects, and

266 nutrient imbalance (*Kohler et al., 2009*; *Shannon, 1997*). Nitrogen fertilization plays a critical

267 role on the growth and development of the many plants, and appropriate N could help to mitigate

268 the damage caused by nutritional imbalances due to saline irrigation (*Al-Rawahy et al., 1992;*

269 *Borzouei et al., 2014; Fan et al., 2015; Duan et al., 2017*). However, related researches showed

270 that rather than N alone, plant growth was significantly affected by the interaction between soil

271 salinity and N (*Papadopoulos & Rendig, 1983*; *Chen et al., 2010*). In this experiment, exogenous

272 N application of N to annual ryegrass seedlings significantly increased the *in planta* N content,

273 plant height and the biomass, but there was a concentration effect. In the absence of exogenous

274 salt, the increment of plant height and biomass was directly proportional to the N level, and

275   peaked at 5.0 mM N. However, after exposure to NaCl, the plant height and the relative increase

276   of biomass peaked at the N level of 2.0 mM (Fig.1). Moreover, ultra-low N seriously inhibited

277   the growth of ryegrass under both control and salt conditions (Fig. S1). These results were

278   consistent with results detected in cotton (*Chen et al., 2010*). Also a previous study on annual

279   ryegrass reported that increasing N application could promote shoot growth under under salt

280   concentrations of 2.0 dS/m (below 20 mM) and 11.2 dS/m (over 100 mM) (*Sagi et al., 1997*).

281   However, we noticed that moderate reduction of N application had a maximum promotion effect

282   on plant growth. The salt concentration used for our treatments are 50 mM and 100 mM, which

283   are different from the salt concentration used in Sagi′s experiments. This difference may be

284   attributed to different culture conditions as well as levels of salinity. In this study, we focus

285   mainly on the optimization of nitrogen application at lower salt concentration. Moreover, N

286   content was also positively correlated with the amount of N applied which peaked at the N level

287   of 5.0 mM without salt treatment. However, under salt treatments, there was no significant

288   difference in N content of ryegrass leaves between 5.0 mM and 2.0 mM N application (Fig. 5B).

289   External cues such as salt can stimulate the production of ROS which can further damage lipids

290   in plant cells (*Kohler et al., 2009*). Accumulation of N-containing compounds has been reported

291   to participate in salt response such as osmotic adjustment and ROS scavenging (*Dluzniewska et*

292   *al., 2007*; *Ehlting et al., 2007*; *Homaee et al., 2002*; *Mansour, 2000*; *Song et al., 2006*; *Sudmalis*

293   *et al., 2018*). In this study, by reducing MDA content and elevating certain antioxidant enzyme

294   activities, moderately low N application could reduce the damage to the membrane of ryegrass

295   seedlings caused by salt stress, especially at relatively low NaCl treatment. Together, these

296   results indicated that saline habitats might change the N requirement of ryegrass seedlings.

297   Excessive or ultra-low N applications both have counter effects on the growth or salt resistance

298   of annual ryegrass under low level of salt stress.

299   Under salt stress, chlorophyll *a* fluorescence transient is a useful parameter for reflecting

300   the primary reaction alternations of PSII, which is more sensitive than photosystem I (PS I). To

301   investigate PSII behaviors in O-J-I-P transient, JIP test is always used to quantify the derived

302   photochemical parameters (*Apostolova et al., 2006*; *Sayed, 2003*; *Stirbet et al., 2014*). In this

303   study, after exposure to NaCl, the nitrogen application level had a significant effect on

304   fluorescent transients, especially the J and P steps (Fig. 2). N deficiency and N over application

305   under salt stress might lead to the photosynthetic electron transport traffic jam, especially beyond

306   Q_A^- (Fig. 2B, C). With the increase of N level, the PI_{ABS}, which could accurately reflect the state

307   of plant photosynthetic apparatus, increased without NaCl treatment (Fig. 2A), indicating that N

308   could promote the primary photochemical reactions of PSII in the waterside. However, under salt

309   stress, excessive or deficient N application slowed down the promotion of the primary

310   photochemical reaction of the PSII (Fig. 2B, C). In addition, the accumulated amount of Q_A^- (V_j)

311   and the relative speed of Q_A deoxidation (M_0) (*Strasser, 1997*; *Strasserf & Srivastava*, 1995;

312   *Force et al., 2003*) of plants grown under moderately low N were lower than those grown under

313   other N conditions, indicating that leaves grown under moderate N level have a higher electron

314   transport rate between Q_A and Q_B, thus reducing the accumulation amount of Q_A^- and increasing

315   the photochemical reaction efficiency (*Allakhverdiev & Murata*, 2004). The increase of Ψ_0 and

316   ΦE_0 of plants grown under 2.0 mM N indicated that leaves use more energy for photochemical

317   reaction and electron-transfer process, thus producing more NADPH for carbon assimilation and

318   confirming that leaves have the optimal energy distribution under certain salt level (*Strasser et*

319   *al., 2004*). The leaves of annual ryegrass grown under 2.0 mM N also had a greater reaction

320   center density RC/CS_0 and higher ET_0/CS_0 but lower DI_0/CS_0 than those grown under other N

321   conditions. This pattern indicated that the specific activity of a unit cross-sectional area of leaves

322   grown under moderately low N was stronger than that grown under other N conditions, reducing

323   the energy burden of a unit reaction center. We also noticed that, under the lowest NaCl

324   treatment (50 mM), the application of 2.0 mM N had the best effect on alleviating salt stress.

325   Under 50 mM NaCl treatment, the physiological indexes of annual ryegrass seedlings seemed to

326   be less affected, and therefore the seedlings might be more sensitive to the promotion of nitrogen

327   application. Thus, we proposed that the optimum amount of N might promote primary

328   photochemical reactions of PSII under certain level of NaCl treatment.

329   After absorption as ammonia, N can be directly assimilated by plants. While after

330   absorption as nitrate, N must first be reduced by nitrate reductase and sub-acid reductase

331   enzymes. NR can reduce nitrate N to ammonium, which has also has important effects on

332   photosynthesis and other processes related to N metabolism (*Xu et al., 2012*). Reports have

333   shown that NO_3^- has a significant effect on the induction of NR expression. From this experiment,

334   under control condition, the level of *NR* gene expression in leaves increased with the increase of

335   N concentration (Fig. 5A, B), which is consistent with the previous reports (*Oaks, 1993*).

336 However, when the seedlings were treated with NaCl, the *NR* expression level was significantly

337 declined at a higher N level (5.0 mM). On the contrary, at a moderately low N level (2.0 mM),

338 the *NR* expression level is relatively low without NaCl treatment, but the degree of reduction is

339 moderate when exposed to NaCl. The *GS* gene expression displayed a similar trend with the *NR*

340 gene under the interaction between salt and nitrogen conditions, indicating an interactive

341 response mechanism between N assimilation-related genes (Fig. S2A). Therefore, it can be

342 observed that moderate N application might help annual ryegrass maintain the expression level

343 of N assimilation-related gene (Fig. 5A) and further maintain the nitrogen content under salt

344 stress (Fig. 5B). However, when excessive N was applied under salt stress, the *NR* expression

345 was significantly increased, indicating that N assimilation was strengthened; it might then

346 compete with photosynthetic carbon for ATP and NADPH and increase the burden of

347 photosynthetic electron transfer. The competition result might lead to a decline in the overall

348 activity of PSII of annual ryegrass (PI_{ABS}) (Fig. 2). Under nitrogen deficiency, the reduced

349 absorption of nitrogen might reduce the consumption of nitrogen assimilation reducing power,

350 most of which are derived from photosynthesis, thus resulting in the accumulation of chloroplast

351 NADPH. The over-accumulation of NADPH could inhibit the photosynthetic efficiency and

352 cause excessive production of ROS (Fig. 3A), leading to increased cell membrane damage,

353 which may in turn lead to reduced photosynthetic efficiency (Fig. 2B, C, D). Nitrogen is also a

354 constituent of chlorophyll which is not only the most important pigment molecules of

355 photosynthesis involved in energy absorption and transmission but also the essential electron

356 mediator during electron transport. Studies showed that the nitrogen content of leaves is constant

357 with the photosynthetic capacity (*Grassi et al., 2005*; *Kattge et al., 2009*). Through this

358 experiment, we can deduce that under different salt stress condition, the appropriate addition of

359 N can increase relative chlorophyll content of plants. However, the relative chlorophyll content

360 is only positively correlated with N levels within a certain range (0.5-2.0 mM) and should be

361 reduced beyond a certain range (Fig. 2D). The moderately supply of N under salt stress increased

362 the content of chlorophyll and might increase the light-harvesting ability, partly contributing to

363 the up-regulated photosynthetic performance index. Based on the above studies, it can be seen

364 that moderately low N application under low level of salt stress might help annual ryegrass

365 maintain the expression level of N assimilation-related gene and then maintain the leaf N content

366 of the plant, which might in turn cause changes in chlorophyll content, further avoiding the

367 negative effect on photosynthetic capacity.

368 **Conclusion**

369   To investigate the possible mechanism of moderately low nitrogen-mediated alleviation of

370 NaCl stress, the degree of lipid peroxidation, antioxidant enzyme activity alternation, changes of

371 photosynthesis performance and nitrogen assimilation were analyzed in this study. In summary,

372 under low salt stress, the demand for N may have declined, while moderately reducing N

373 application could help to alleviate the salt-induced damage in annual ryegrass mainly by

374 alleviating the damage caused by ROS and promoting the performance of photosynthesis and

375 nitrogen metabolism. Further, in order to enhance plant growth and increase nitrogen use

376 efficiency, the optimum application of nitrogen fertilizer needs to be controlled to match the

377   plant needs at each growth stage and to adapt to different saline environment.

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381   **Reference**

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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   50   100 mM   0   50   100 mM   0   50   100 mM NaCl

B

C
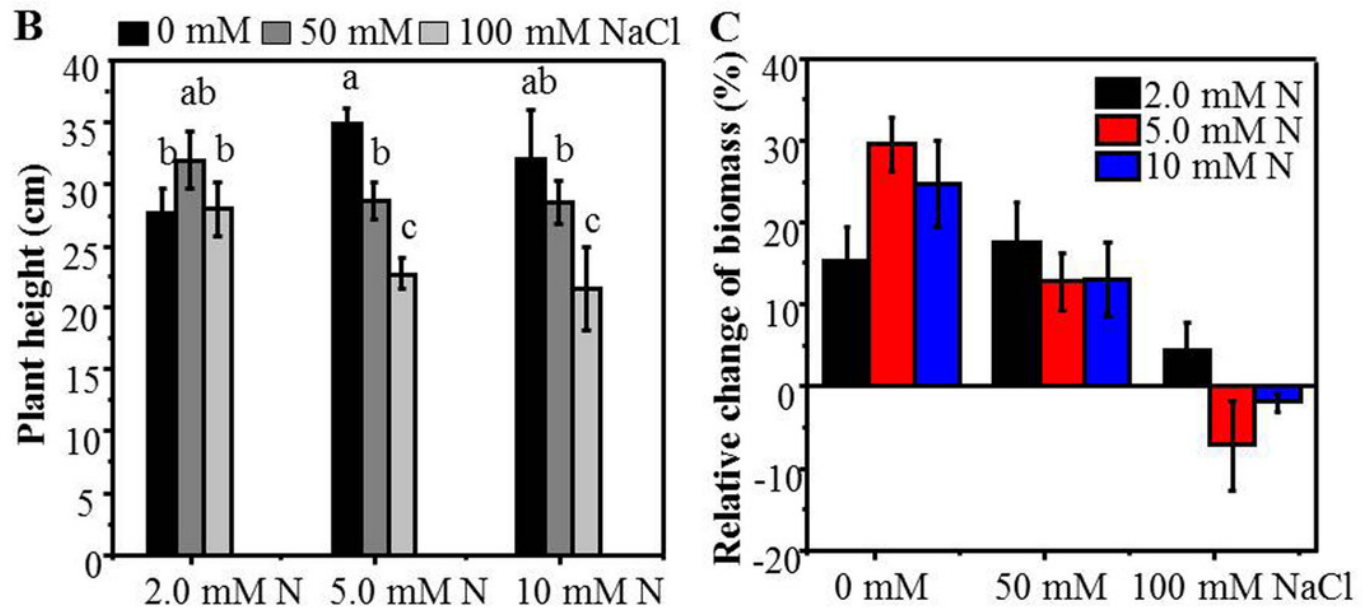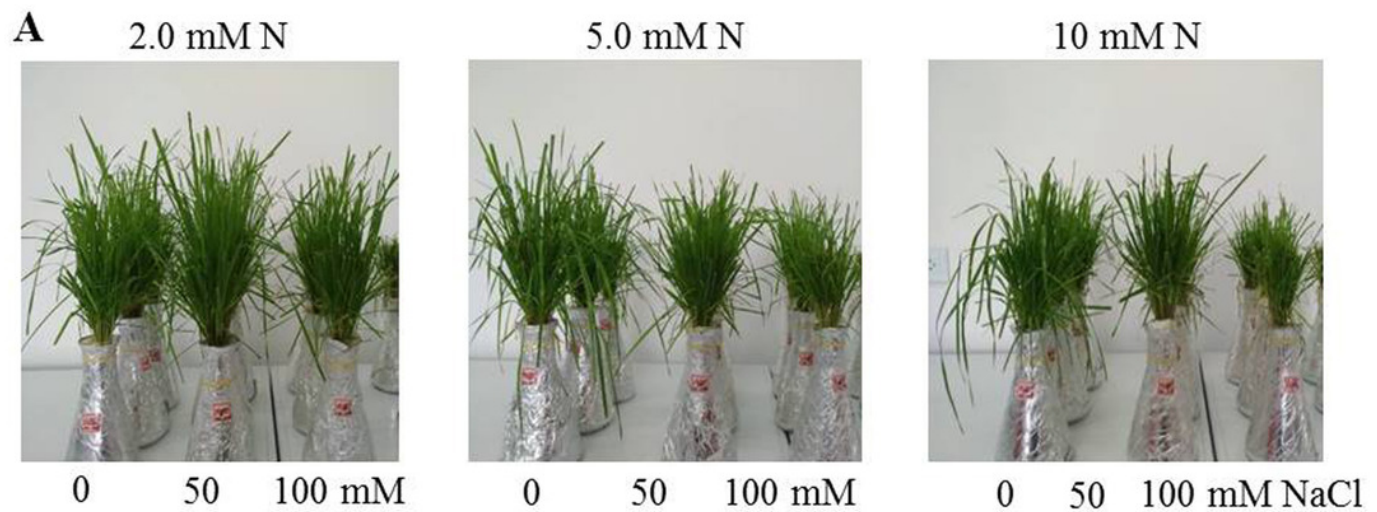
Figure 2

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.
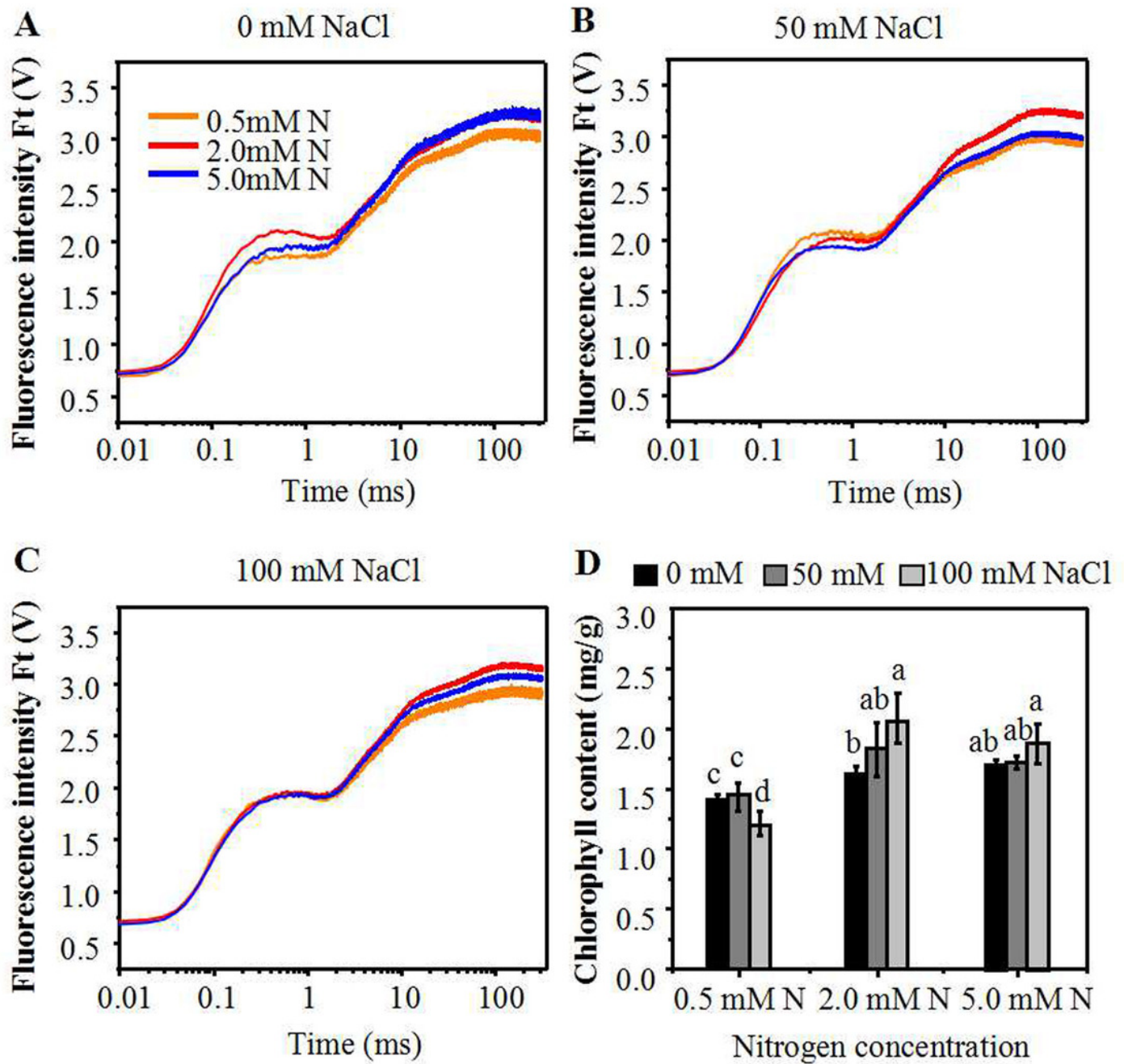
Figure 3

"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 significant differences of Chlorophyll fluorescence parameters between different N levels at $P < 0.05$ by Student's t–test under 0, 50, 100 mM NaCl respectively.
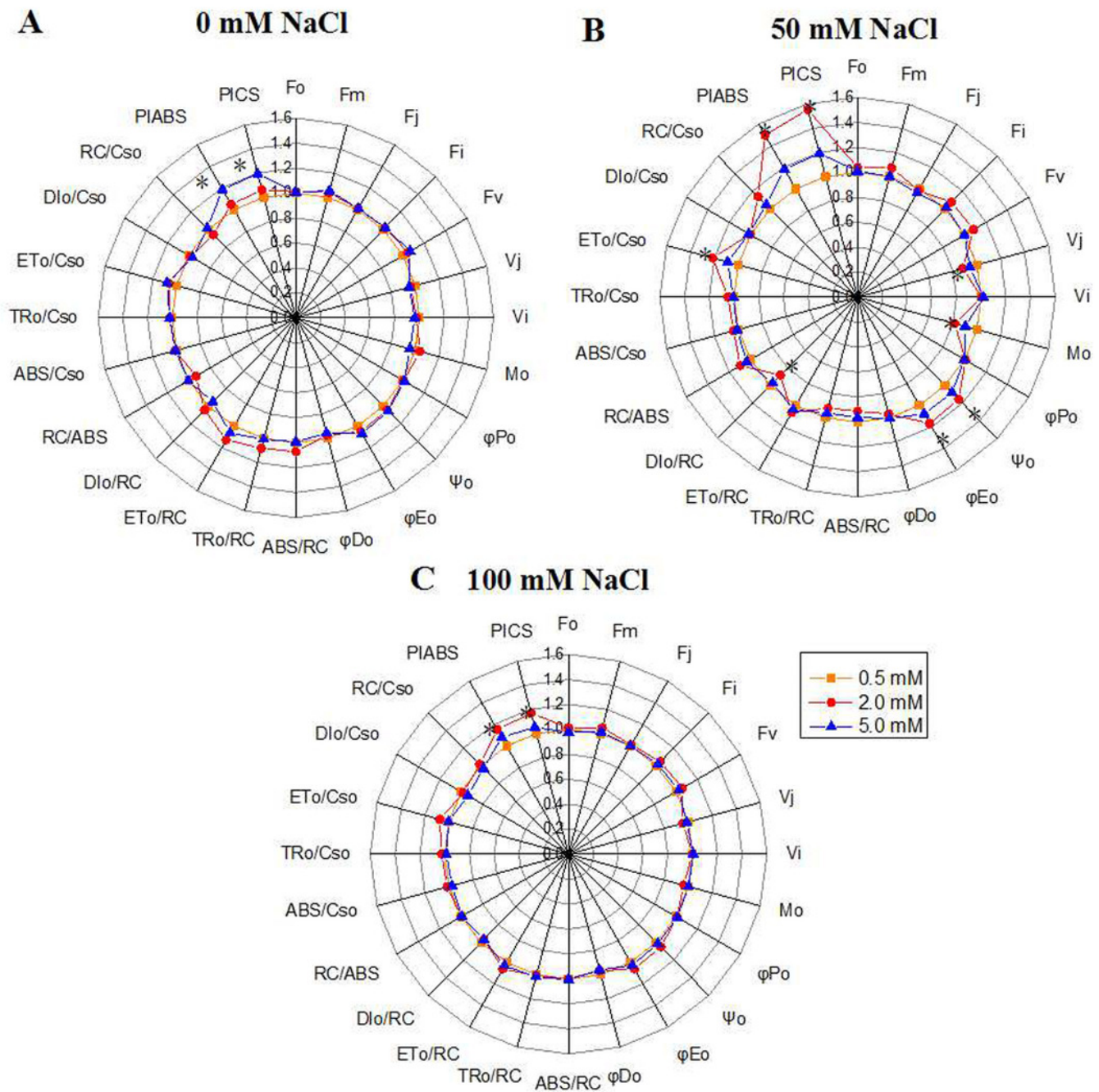
Figure 4

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.
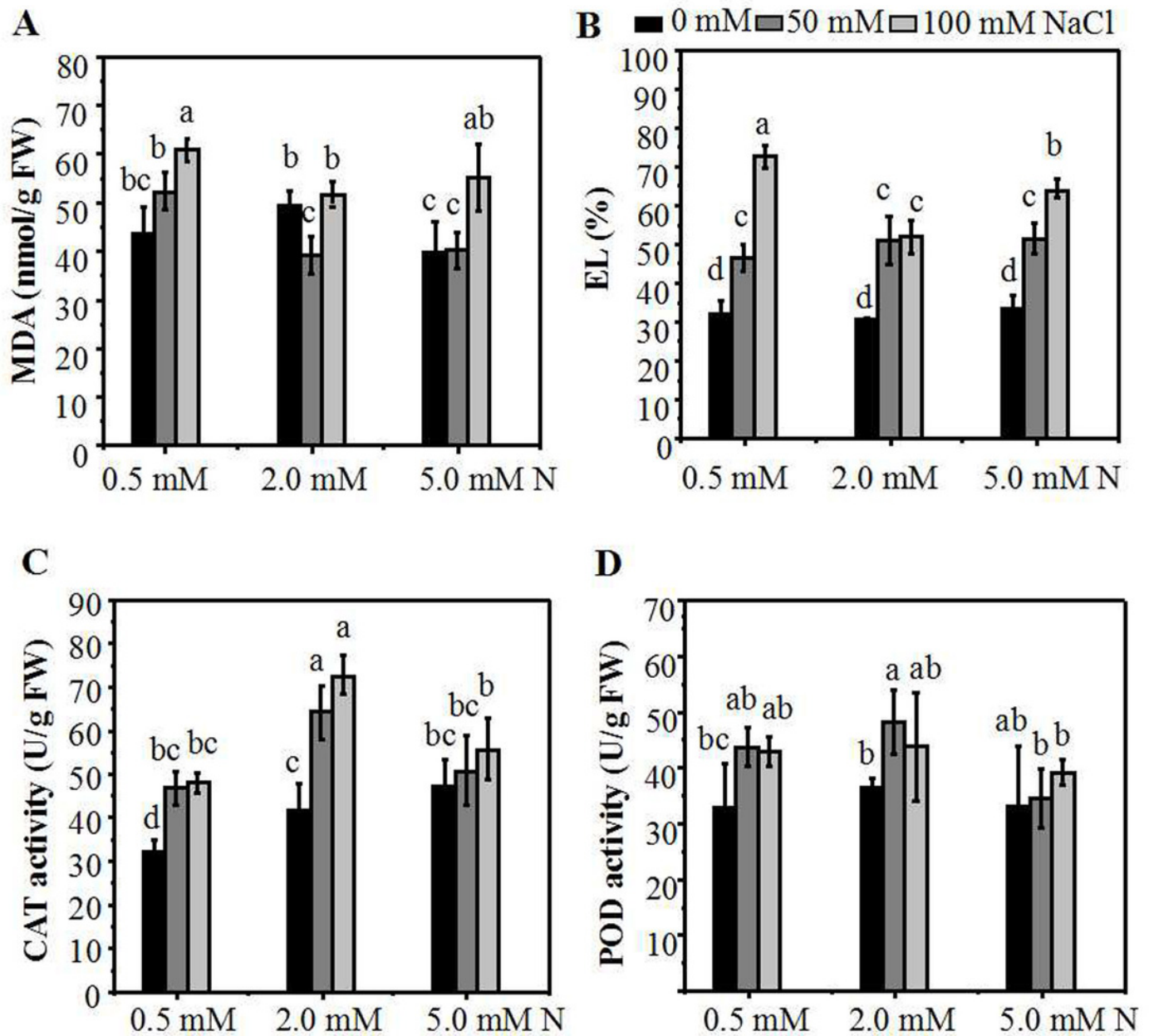
Figure 5

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.