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Interactive salt—Alkali stress and exogenous Ca^{2+} effects on growth and osmotic adjustment of *Lolium multiflorum* in a coastal estuary

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ABSTRACT

Salt and pH levels in coastal estuaries are very high. In our study, different degrees of interactive salt – alkali stress were simulated to determine the effects of interactive salt – alkali stress on the growth and osmotic regulation of *Lolium multiflorum* (group A experiment). In addition, Ca^{2+} solutions were prepared to examine the mitigative effects of exogenous Ca^{2+} on interactive salt – alkali stress (group B experiment). In group A, five salinity levels (0‰, 5‰, 10‰, 15‰, 20‰) were set. Each salinity level was further categorized at three pH levels: 7 ± 0.12, 8 ± 0.22, and 9 ± 0.16. In group B, five Ca^{2+} levels (0, 2, 4, 6, and 8 mM) were added to the experiment. Damage of *L. multiflorum* by interactive salt – alkali stress was more pronounced than by single salt stress. The tolerable range was defined for pH 7 and salinity ≤10‰, and pH 8 and salinity ≤5‰, respectively, when the leaf relative electrical conductivity was not significantly increased, the chlorophyll content and root vigor were not significantly reduced, and the mortality rate of *L. multiflorum* was low. However, growth was decreased by more than 90% (at pH ≥ 8 and salinity ≥15‰), and osmotic regulation of *L. multiflorum* was drastically impaired beyond the tolerance range. Under single salt stress, the content of proline, Na^+/K^+ ratio and Cl^- content were lower than that under interactive salt – alkali stress, while the contents of Ca^{2+} and Mg^{2+} were higher. The optimum exogenous Ca^{2+} concentration was 6 mM for *L. multiflorum*. At this concentration, leaf relative electrical conductivity, proline content and Na^+/K^+ were lowest, and chlorophyll content, root vigor and Mg^{2+} content were highest. Overall, *L. multiflorum* could tolerate a certain degree of interactive salt – alkali stress, which can be beneficial for the ecological restoration of coastal waters.

1. Introduction

Water in coastal estuaries contains high quantities of neutral salts, such as NaCl, and other alkaline salts, including Na_2CO_3 and $NaHCO_3$, which are influenced by seawater. Alkaline salts contain $HCO_3{}^-$ and $CO_3{}^{2-}$, which increase pH of the water. High salinity and pH levels damage the plants in these ecosystems; as a consequence, plant diversity becomes scarce and landscapes deteriorate. However, most studies focus on mechanisms to combat single salt stress (Wang et al., 2014; Jamali et al., 2015; Brown et al., 2006; Ochoa-Alfaro et al., 2008), single alkali stress (Brand et al., 2002), or on comparative effects of salt-stress and alkali-stress on plants (Chen et al., 2011; Guo et al., 2015). These single factors, however, are inconsistent with actual conditions in coastal estuaries, where salt and alkali exist at the same time. Studies showed that interactive salt – alkali stress differs from individual salt or alkali stress (Shi and Sheng, 2005; Shi and Yin, 1993; Li et al., 2010; Yang et al., 2011). The influence of high pH is significantly enhanced as salinity increases (Chen et al., 2015; Li et al., 2010). However, the range of salt, alkali or interactive salt – alkali stress was narrow in these studies. The highest salinity in the research of Yang et al. (2008a) was 9.3‰. In the study on the relationship between plant plasma membrane ultrastructure and NaCl stress by Gupta (2007), the highest salinity was only 11.7‰. Watanabe et al. (2000) studied the relationship between salt stress (the highest salinity was set as 4.4‰) and proline. However, under a wide range of interactive salt – alkali stress (salinity 0–20‰, pH 7–9), the growth and physiological responses of plants are still unclear.

In extreme circumstances, when the plants are badly damaged, artificially adding certain exogenous substance can improve the

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ability to adapt to the environment (Liu et al., 2014; El-Hamdaoui et al., 2003). Under salt stress, these substances mainly include inorganic ions (such as Ca^{2+}, K^+) (Harper et al., 2004), organic solvents (such as trehalose, spermidine) (Abdallah et al., 2016; Li et al., 2016) or microbes (Garg and Pandey, 2016) to alleviate the damage of plants. Since Ca^{2+} is an economic and environment-friendly inorganic ion, many studies have shown that Ca^{2+} is a relief for the growth of plants under salt stress (Khan et al., 2010; Khan et al., 2012). Knight (1999) thought that Ca^{2+} can maintain the integrity of plant cell structure and function under salt stress. Guimaraes et al. (2011) found that Ca^{2+} can partially offset the toxic effects of Na^+ under salt stress. Yin et al. (2014) found that Ca^{2+} can increase plant biomass under NaCl stress. But currently there is a lack of studies on Ca^{2+} relief for plants at interactive salt – alkali stress. In real ecosystems, the implementation of Ca^{2+} for easing salt – alkali tolerance of plants is not easily feasible, but it plays an important role to study the salt – alkali tolerant mechanism of plants and investigate the potential of Ca^{2+} fertilizers to improve plant salt-tolerance.

Lolium multiflorum Lam. is a dominant species which can survive in the salt-alkali environment (salinity 0–20‰, pH 7–9) of the Licun River estuary region, Qingdao, China. Growth of most plants will slow or even stop in this salt–alkali environment, especially during the winter; however, *L. multiflorum* is capable of retaining its vitality. Thus, *L. multiflorum* can not only improve the estuary landscape environment, but can be also used as a forage grass when other plants suffer from winter kill. Yet the interactive salt – alkali tolerance mechanism of *L. multiflorum* is unclear. Therefore, this study chose *L. multiflorum* as the research object to investigate the mechanism of interactive salt – alkali tolerance, and the mitigative effects of exogenous Ca^{2+} on the salt – alkali stress. This study mainly aimed (1) to identify the damage of a single salt stress and interactive salt – alkali stress conditions on the growth of *L. multiflorum* seedlings, (2) to describe mitigative effects of exogenous Ca^{2+} on the growth and physiological responses of *L. multiflorum* under interactive salt – alkali stress.

2. Materials and methods

2.1. Plant materials

Lolium multiflorum is an herbaceous perennial grass that is grown as an ornamental grass. It can be used as a cover crop to remove nitrogen and phosphorus.

Seeds of *L. multiflorum* with similar size and weight were placed in Petri plates (9 cm) with two layers of filter paper. Distilled water was poured to wet the paper for facilitating seed germination. When the shoots of the seedlings were 5 cm long, the seedlings were transferred to 250 mL plastic pots (71 mm diameter and 97 mm high) with 200 mL of Hoagland nutrient solution. The seedlings were placed with plastic foam board. Seedlings were used for the experiment when the shoot grew up to 10 cm height and formed new roots. The total fresh weight of every plastic pot was 15 g.

2.2. Experimental scheme

2.2.1. Experimental design and procedures

The experiment was divided into two parts, group A and group B. In group A, the growth and physiological response of *L. multiflorum* under interactive salt and alkali stress were examined. In group B, the mitigative effect of exogenous Ca^{2+} on interactive salt and alkali stress influencing *L. multiflorum* was analyzed. The following experimental designs were used.

Before the two experiments were performed, water quality was investigated in the Licun River estuary (Qingdao, China). Results showed that the salinity of the water of Licun River estuary is as high as 19.3‰, pH 9.2, and the content of total inorganic nitrogen and total phosphorus is as high as 8.5 mg/L and 5.8 mg/L, respectively, more serious than the worst class water of Environmental Quality Standards for Surface Water(GB3838-2002)of China (supplementary material).The components of the test solution to cultivate the plants were chosen based on these results (Table 1).

2.2.1.1. Group A. The stress conditions were applied in a factorial design: five levels of salinity (0, 5, 10, 15 and 20‰); under each salinity set three levels of pH (7, 8 and 9), totalling 15 experimental treatments. Each experimental treatment was applied to independent replicates of 3 different pots (several plants were cultivated in one pot). NaCl was used to regulate the salinity levels; Na_2CO_3 and $NaHCO_3$ were used to adjust pH 8 and 9 at the following proportion: 0:1 or 1:9 according to Li et al. (2010). In total salinity ranges, the treatments of pH 7 were single salt stress, the treatments of pH 8 and pH 9 represented the interactive salt – alkali stress. Thus, pH stress refers to alkali stress. A treatment of pH 7 and salinity of 0‰ was the control group. Salinity was increased by 5‰ and pH was increased by 1 unit daily to prevent salt shock effects until the desired value was reached. Water lost through evaporation was supplemented daily with distilled water using a weighing method.

2.2.1.2. Group B. On the basis of the results obtained from group A, we selected pH 8 and 5‰ salinity as the salt – alkali condition of group B. The Ca^{2+} concentration was considered a variable in the test solution. The total concentration of exogenous Ca^{2+} was set at five levels: 0, 2, 4, 6, and 8 mM (according to results from the investigation in Licun River). Similar to group A, the experimental treatments of group B were repeated 3 times. The test solution (Table 1; but without Ca^{2+}) and $CaSO_4$ were used to prepare different Ca^{2+} levels. A treatment of Ca^{2+} of 0 mM was the control group.

For both groups, the experimental plastic pots were placed in a lab with natural sunlight. Temperatures were 20–25 °C during the day and 15–18 °C at night. The experiment ran over 14 days.

2.2.2. Analytical methods

The plants were harvested after 2 weeks and rinsed with distilled water. The remaining water on the surface of the plants was blotted with filter paper. Portions of the fresh samples were obtained to measure fresh weight (FW) and physiological parameters (leaf relative electrical conductivity, proline content, chlorophyll content, and root vigor). The remaining samples were dried to a constant mass at 75 °C (dry weight, DW) and used to measure ion contents (Na^+, K^+, Cl^-, Ca^{2+}, and Mg^{2+}).

The plant mortality rate was denoted by the ratio of the number of dead plants and the total number of plants. The DW increment, which means the plant dry weight at the end of the experiment minus the start of the experiment, was quantified via weighing the plants with a precision of 0.0001 g. The water content (WC) was calculated as follows: $WC\ (\%) = [(FW - DW)/FW] \times 100$ (Kingsbury

Table 1
The components of the test solution used to cultivate the plants.

Constituents	Ammonium nitrogen	Nitrate nitrogen	Total inorganic nitrogen	Total phosphorus	Ca^{2+}
Concentration ($mg\,L^{-1}$)	3.6 ± 0.1	2.7 ± 0.1	6.3 ± 0.2	3.4 ± 0.1	40 ± 1.1

Data represent means ± SE (n = 3).