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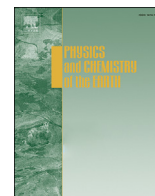
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ABSTRACT

Coastal eutrophication has become a driver of coastal wetlands loss. Eutrophication caused by the increase of nitrogen content was one of the most main reasons. We analyzed that exogenous ammonium nitrogen (EAN) of different concentration influenced on the growth and physiology of *Spartina alterniflora* Loisel (*S. alterniflora*) through simulated conditions. The results showed that growth of the root system largely depended on the environment conditions around *S. alterniflora*. Higher nitrogen concentration promoted aboveground biomass and increased plant height. On the other hand, as the increase of growth period, higher nitrogen concentration could inhibit the elongation growth of root and reduce the underground biomass. We showed that activity of POD, SOD and MDA content changed in an upward trend along with the increased nitrogen level. There was a significant positive correlation between H^+ flux and NO_3^- flux ($r = 0.601, P < 0.01$), and a significant negative correlation between H^+ flux and NH_4^+ flux ($r = -0.713, P < 0.01$) within 1.5 mm from the root tip of *S. alterniflora*. Efflux and influx of ions were associated with changes of nitrogen levels. This research will provide data supporting for coastal wetland restoration of biodiversity reduction caused by coastal eutrophication.

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1. Introduction

Coastal wetlands are the most active interface between land and oceans, and they are also one of the most valuable ecosystems on Earth (Cui et al., 2016). They have special ecological value and resource potential (Zhao et al., 2016). However, many coastal wetlands are suffering from severe physicochemical changes and are often fragile and sensitive areas (Zofia et al., 2014). As the world's population expands and with rapid economic development, a large number of pollutants are entering coastal areas, which threaten the water environment and have negative effects on primary productivity and biodiversity in coastal ecosystems (Thomas et al., 2016). Coastal eutrophication caused by excessive nutrient inputs and accumulation potentially impact on the coastal environment and coastal species, and it has become one of the most important questions of coastal zone research (Bai et al., 2015; Nathaniel, 2014).

As a major producer in coastal wetland ecosystems, *S. alterniflora* plays a key role in ecosystem services and capturing the sediment to protect the coastal wetlands (Mozdzer et al., 2011). Over the past few years, an accelerated global nitrogen cycle has

caused harmful algal blooms and hypoxia from land to coastal marine ecosystems. Our study area chose in the Yellow River Delta. Previous researches have occurred in the area (He et al., 2012; Tian et al., 2009; Lu et al., 2016; Gao et al., 2016). Previous studies on *S. alterniflora* mainly focus on community competition relationship and wetland structure (Fagherazzi et al., 2012), but the way *S. alterniflora* nitrogen absorption and translocation is poorly understood. It is believed that rising concentrations of ammonium nitrogen can cause coastal eutrophication (Linda et al., 2012). Concentration of exogenous ammonium nitrogen rises whether the change affects the growth and physiology of *S. alterniflora*. It is very important that the effect of nutrient on *S. alterniflora* will directly bring the change of coastal wetland ecosystem.

This investigation applied the Scanning Ion-selective Electrode Technique (SIET) to attempt to reveal how *S. alterniflora* roots absorb nutrition and to explore the impact of EAN on *S. alterniflora*. So far, this technique has been used in such fields as material, life, and environmental sciences, and it has been particularly well applied in environmental science because of its high sensitivity (Li et al., 2012; Sun et al., 2009). We undertook to investigate how EAN at different concentrations affected the growth of *S. alterniflora* and nutrition absorption by its roots and leaves. These findings can serve as a theoretical basis for coastal wetland restoration under coastal eutrophication.

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2. Material and methods

2.1. Plant material and grow condition

A. The impact of EAN on the whole *S. alterniflora* growing season. *Spartina alterniflora* was sampled in the yellow river delta. Study area of sampling was introduced in the past study (Zhang et al., 2016a,b). After *Spartina alterniflora* was sampled, we took *Spartina alterniflora* to the Yellow River delta management stations for pre-incubated. The experiment was conducted in a greenhouse of Yellow River delta management stations. Four glass vats (1 m × 1 m × 1 m) were prepared with nine built-in earth holders (size: 15 cm × 15 cm × 20 cm), in which the same weight of self-made soil was placed. The specific procedures for the experiment are as follows: EAN was added to the soil samples at 0 mg/kg, 100 mg/kg, 200 mg/kg, and 400 mg/kg ammonia and the soil was mixed. Three replicates were taken in each processing. EAN was constituted by NH₄Cl. *S. alterniflora* was cut into 10 cm long telomes and planted in the soil samples containing the different ammonium concentrations. *S. alterniflora* was grown on June 28, 2015 and the leaves and roots were analyzed on July 28, August 28, and September 28, respectively.

2.2. Ion flux experimental procedures

When the microelectrode of the Bio-IM non-destructive micro-measurement system and Scanning Ion-selective Electrode Technique (SIET) is moved above the sample surface in a vertical position, it can detect the movement direction and speed of H⁺, NH₄⁺, and NO₃⁻ fluxes at a distance of 15 μm above the surface (Luo et al., 2013). The microelectrode is shaken vertically above the root surface and the ion flux data are obtained with the assistance of influx software. The H⁺ electrode solution (pH = 7.0) was prepared from 15 mM NaCl and 40 mM KH₂PO₄; the NH₄⁺ electrode solution was prepared from 100 mM NH₄Cl; and 10 mM KNO₃ was used as the NO₃⁻ electrode solution (Xu et al., 2006).

2.3. Statistical analysis

All treatments were assigned randomly. The ion flux differences

in various parts of the root were evaluated by SPSS 13.0 and GraphPad prism 5.0. Root length was measured using a ruler. Analysis of variance (ANOVA) was conducted to compare the biomass, plant height and root length in different NH₄Cl treatment. The statistical significance for tests was set at P < 0.05 and test with post hoc contrasts by Student-Newman-Keuls test.

3. Results

3.1. The influence of EAN on the growth and physiology of *S. alterniflora*

The different EAN concentrations affected *S. alterniflora* biomass and height during its growth stage (Fig. 1). Identical letters demonstrated no significant difference, and bars marked with different letters indicated that there was a significant difference when α reached a level of 0.05. Multiple comparisons were made during the same period by comparing the four separate treatments. Error bars showed the standard error of the means (n = 4). Fig. 1 showed that *S. alterniflora* could grow normally in the different ammonium nitrogen treatments. In different growth stage, plant biomass and height had significant differences. Aboveground biomass and plant height increased as growth time, and they reached the maximum in the late stage. Compared to the initial stage, plant height improved 27%, 35%, 42% and 53% with the increasing nitrogen treatment concentration in the late stage. In the late stage, the aboveground biomass of *S. alterniflora* growing in soil with 200 mg/kg EAN and with 400 mg/kg EAN increased to triple as compared with the initial stage of growth. We found that there were few differences in plant height and aboveground biomass during the early stage of growth, in the middle period, there was no significant difference in biomass between *S. alterniflora* grown in soil without and with 100 mg/kg of EAN. However, there were significant differences between the 200 mg/kg and 400 mg/kg treatments (P < 0.05). However, during the last phase of growth, the plant height and aboveground biomass of *S. alterniflora* both increased significantly (P < 0.05). Rising treatment concentration also improved the aboveground biomass and plant height. In the middle of growth, underground biomass and root length of *S. alterniflora* grew much better than during the other growth

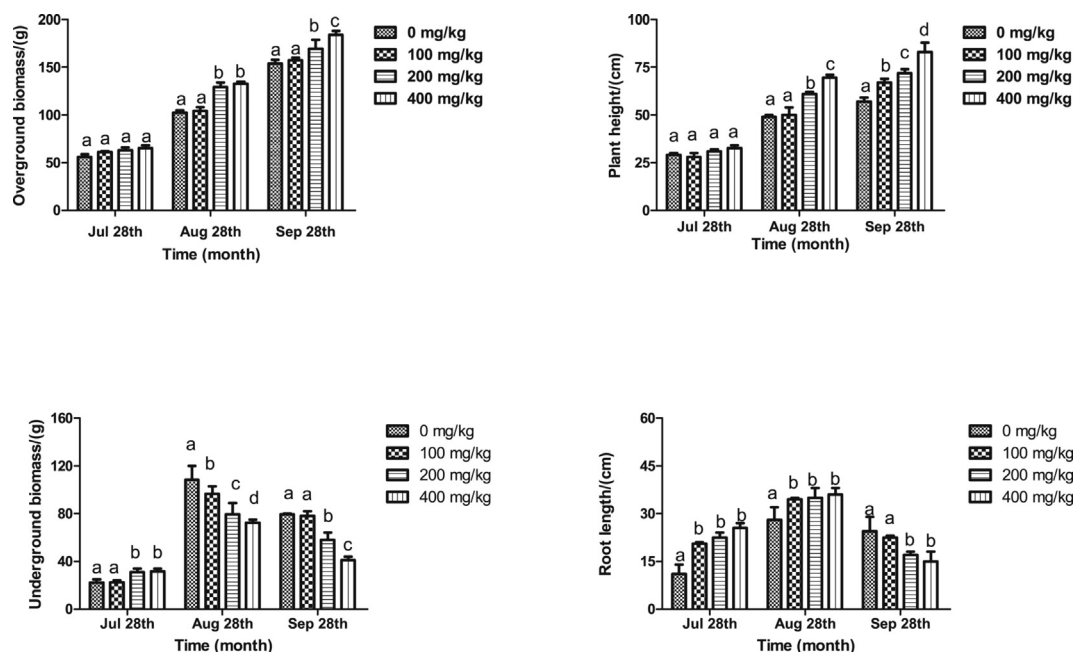


Fig. 1. Biomass, plant height and root length along *S. alterniflora* in different NH₄Cl treatment.

stages. At the same time, underground biomass gradually dropped with the increasing treatment concentration. In the late stage, higher treatment concentration restrained root length. Therefore, increasing nitrogen treatment concentration promoted plant height and aboveground biomass growth and restrained the root length and underground biomass of *S. alterniflora*.

In this research, EAN of different concentration was added to test on *S. alterniflora*. R and L represented roots and leaves of plant respectively. Oxidase system and MDA content of roots and leaves of *S. alterniflora* were illustrated in Fig. 2. From it, we can see that activity of POD, SOD and MDA content changed in an upward trend in roots and leaves of *S. alterniflora*. But different nitrogen treatment concentration led to slight differences in their change tendency. In contrast to its roots, POD, SOD and MDA were slightly weaker than those in the leaves of *S. alterniflora*. The roots of *S. alterniflora* were under more stress than its leaves.

3.2. Impact of different nitrogen treatment concentration on H^+ ion flux of *S. alterniflora*

We carried out a further analysis of roots. The H^+ flux changes in the root tips of *S. alterniflora* with different EAN concentrations were shown in Fig. 3. The range over the horizontal axis was exocytosis and below it represented uptake. The figures with A, B, and C represented different periods of *S. alterniflora* growth, which were as follows: A stood for the initial stage of growth, B

stood for the middle stage, and C stood for the last stage. The dividing lines between these phases were July 28, August 28, and September 28. Identical letters demonstrate that only a minor, non-significant difference exists, and bars marked with different letters indicate that there was a significant difference when α reached a level of 0.05. Multiple comparisons were made between the four separate treatments at the same time. At the early stage of growth, as the EAN concentration rose, the root efflux tended to increase. A comparison between *S. alterniflora* without EAN and those planted in soil with an EAN of 400 mg/kg, showed that H^+ increased by 62.31%, whereas the leaf activity basically remained unchanged. At the middle stage, as the EAN concentration rose, the H^+ flux of the leaves gradually increased while that of the root only went up by a small amount. Over the whole growth period, ion pumping by the roots was higher than by the leaves, except at 400 mg/kg during the middle stage. High EAN concentrations affected the ability to discharge H^+ via the roots during the middle stage.

The H^+ flux was measured in the root and various parts of root within 2.0 mm of the tip. Change was noticeable within 0.5 mm of the root tip. Beyond the root tip, H^+ efflux tended to be more stable. In the early stage of growth, the H^+ efflux of *S. alterniflora* without EAN differed considerably from *S. alterniflora* treated with EAN at various concentrations. At the same test point, the higher the EAN concentration was, the more obvious was the absorption. Compared with the initial stage of growth, the root tips growing in 400 mg/kg EAN during the middle stage of growth were significantly different from those treated with the other EAN concentrations. H^+ inflow appeared at several test points within a 0.5 mm length starting from the root tip. During the last stage of growth, H^+ flux tended to become smaller and more stable. The 0.5 mm length, starting from the root tip, was the most sensitive area for H^+ absorption and efflux. The further away from the root tip, the more stable the ion flux was. The root tip is the most sensitive part and influences overall root growth.

3.3. Impact of different nitrogen treatment concentration on NO_3^- ion flux of *S. alterniflora*

Changes of NO_3^- flux at the root and root tip of *S. alterniflora* from above to below in Fig. 4. The monitor of *S. alterniflora* root was within 0.5 mm away from root tip. During the early stage of growth, NO_3^- efflux took place at the root tip without ammonium nitrogen treatment, whereas NO_3^- inflow occurred at the root tip treated with EAN. Thus a significant difference existed between the two ($p < 0.05$). During the middle stage of growth, NO_3^- inflow took place at the root tip when *S. alterniflora* was exposed to EAN and the tendency became increasingly stronger ($P < 0.05$) as the concentration rose. In contrast, NO_3^- efflux occurred at the root tip when *S. alterniflora* was treated with EAN, but the tendency became weaker as the concentration rose. During the last phase of growth, NO_3^- flux by the roots treated with EAN tended to be stable and there was no significant difference at the 0.05 level between treatments. Therefore, EAN can change NO_3^- uptake and exocytosis along the root tip. In the initial stage of growth, EAN can facilitate NO_3^- absorption by the root tip. As time progressed, it could convert NO_3^- inflow to efflux, which was in contrast to the situation without EAN.

3.4. Impact of different nitrogen treatment concentration on NH_4^+ ion flux of *S. alterniflora*

NH_4^+ flux changes in the root and at other test points showed in Fig. 5. During the early stage of growth, NH_4^+ uptake took place at the root tip when no EAN was added. However, with higher the

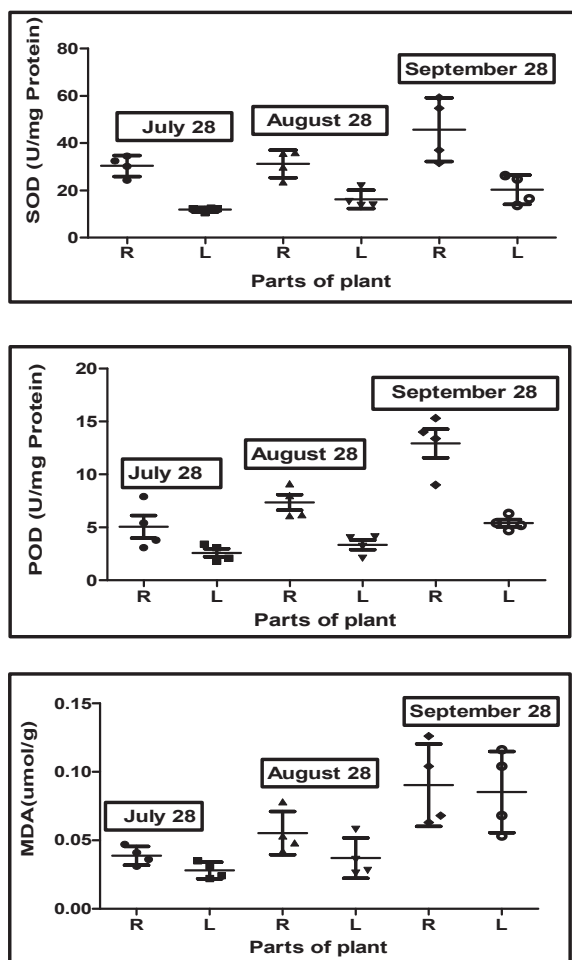


Fig. 2. Effects of different NH_4Cl treatment on the peroxidase, superoxide dismutase activity and malondialdehyde.

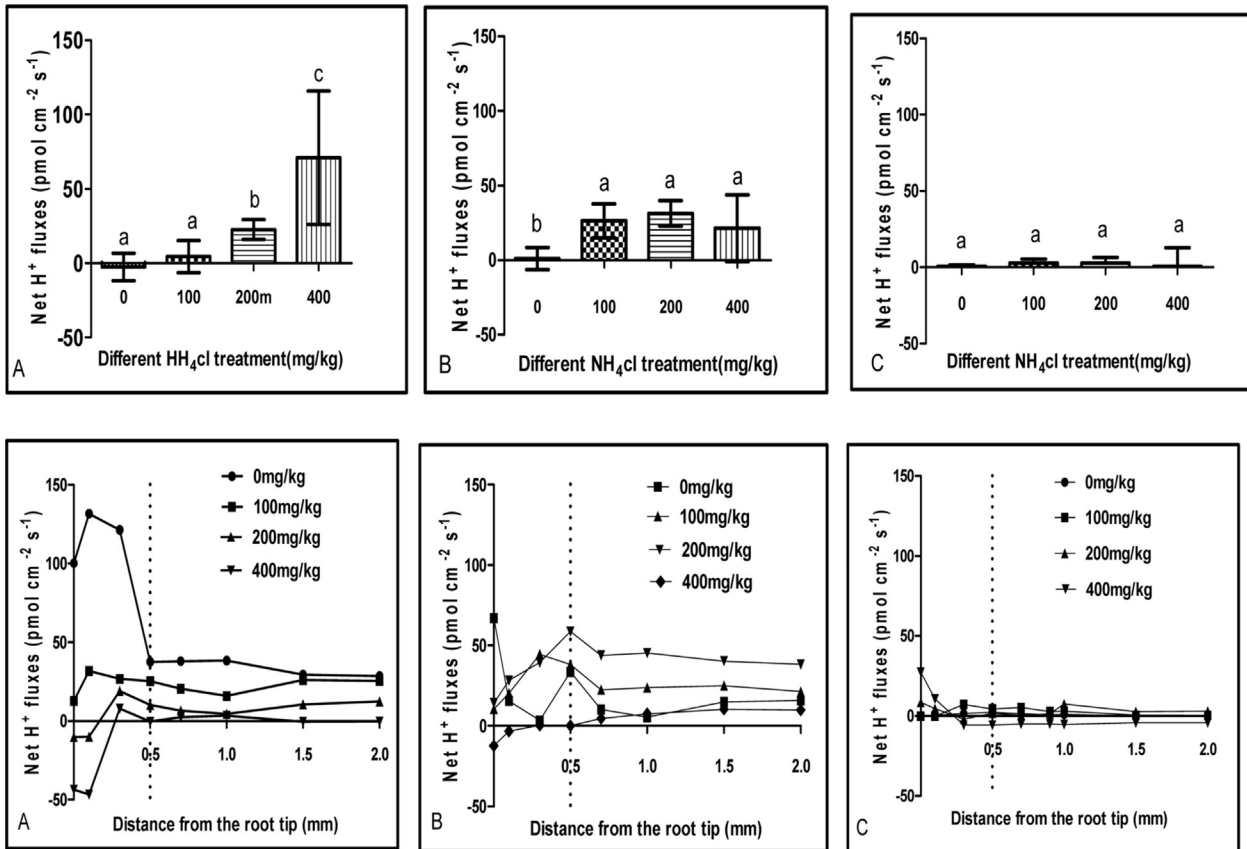


Fig. 3. H^+ flux along roots and root tips of *S. alterniflora* in different growth phase.

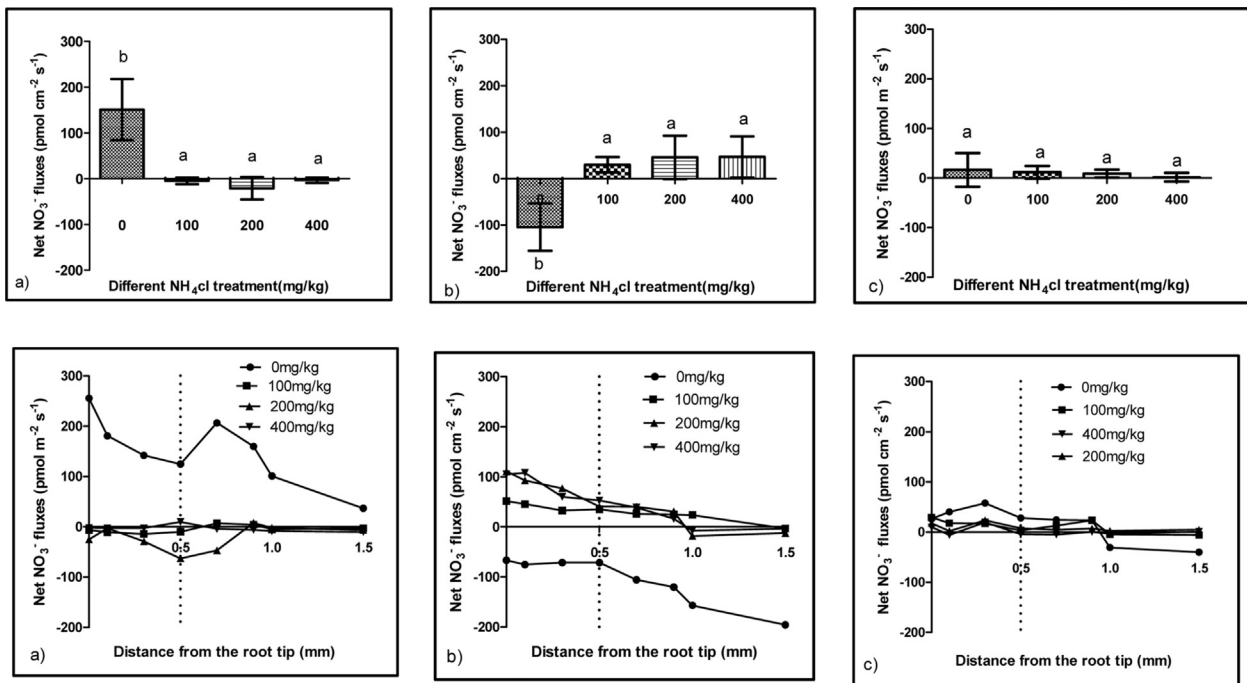


Fig. 4. NO_3^- flux along roots and root tips of *S. alterniflora* in different growth phase.

EAN concentration, NH_4^+ efflux was more intensive. During the middle stage of growth, NH_4^+ inflow took place at all the points except no EAN at 0.5 mm from the root tip. At the other points, NH_4^+

uptake increased gradually as the concentration rose. During the last phase of growth, the plant showed NH_4^+ efflux. EAN is translated NH_4^+ uptake into NH_4^+ efflux at the root tip.

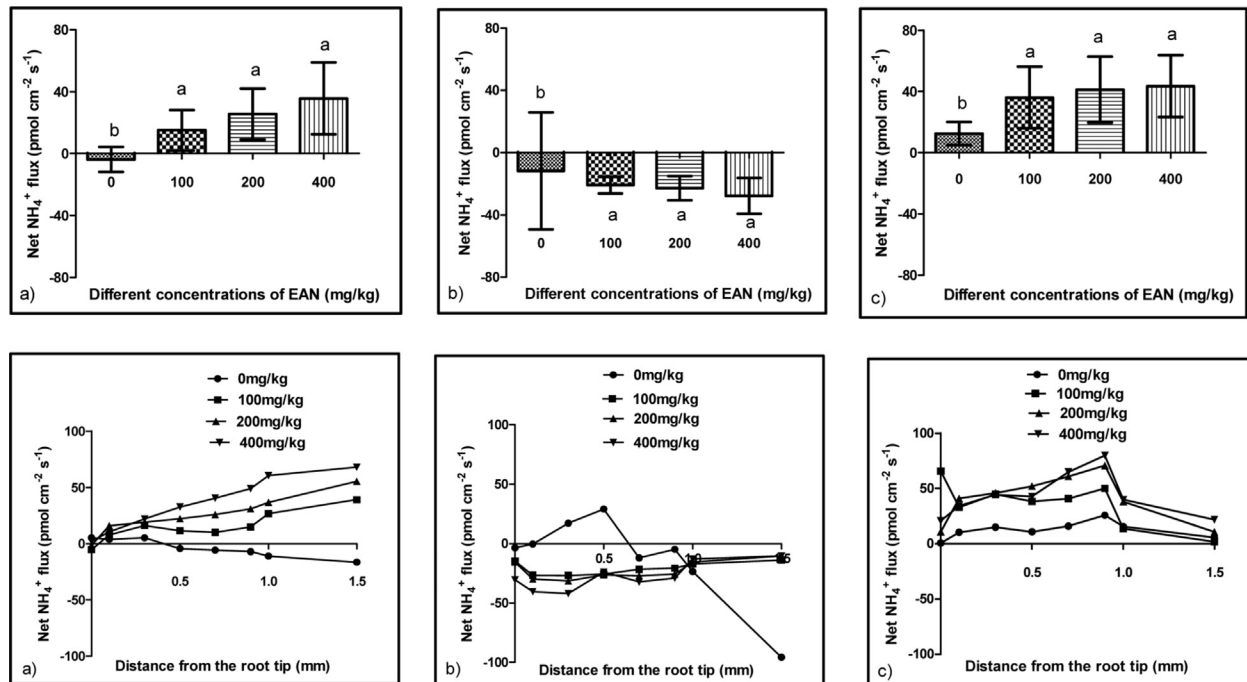


Fig. 5. NH_4^+ flux along roots and root tips of *S. alterniflora* in different growth phase.

4. Discussion

We detected changes of ion fluxes at the root of *S. alterniflora* with EAN of different concentrations. Changes affected by EAN concentration were obvious within the range of 0.5 mm from the root tip. Earlier research had found that absorption sites of nitrogen closed the root tip, and degree of absorption decided by nitrogen concentration (Babourina et al., 2007). The root elongation was consistent with the research findings concerning Arabidopsis (Barth et al., 2010). These results indicated that growth of the root system of *S. alterniflora* largely depended on the environment conditions. Higher EAN could promote aboveground biomass and increase plant height in this study. On the other hand, higher EAN could inhibit the elongation growth of root tips, and affected adversely the growth of the root system.

EAN resulted in some changes of physiological function of the *S. alterniflora*. According to some research, higher concentration ammonium nitrogen induced changes of antioxidase in plants, and activity of SOD and POD increased with the rise of concentration of ammonium nitrogen (Wang et al., 2008). Higher activity of SOD and POD effectively eliminated active oxygen radicals and reduced harm caused by peroxidation of the plant membrane (Jorge and Stephan, 2007). Through correlation analysis of MDA content of *S. alterniflora* root system and H^+ flux at root tip, there was extremely significant negative correlation between MDA content of *S. alterniflora* root and H^+ flux at the root tip ($r = -0.871, p < 0.01$). This change may be related with growing environment of the plants (Diggelen et al., 2015). In stress condition, the rate of oxidative phosphorylation slowly released H^+ and enzymatic reaction lost balance. The results lead to the increasing MDA content and more serious stress threaten to the plant (Li et al., 2010). MDA was one of peroxidized products of plant adipose membrane when the plant grew old or stress environment, while its content indirectly indicated how seriously the membrane had been damaged (Corstanje et al., 2006). The change of H^+ ion flux at the root reflected the degree of *S. alterniflora* growth and indicated to what degree the plant had been threatened by nutrient stress. Thus it also reflected some

changes of coastal wetland ecosystem. This research found that a similar tendency appeared between H^+ flux and NO_3^- flux. There was a significant tendency positive correlation between H^+ flux and NO_3^- flux ($r = 0.601, P < 0.01$). There was a negative correlation between NO_3^- flux and NH_4^+ flux ($r = -0.607, P < 0.01$) and H^+ flux showed a negative correlation with NH_4^+ flux ($r = -0.713, P < 0.01$).

Our research group has done many researches on nitrogen (Wang et al., 2016; Huang et al., 2014; Gao et al., 2014; Zhao et al., 2015; Zhang et al., 2016a,b). EAN has attracted the attention of a great height. EAN caused changes of ion fluxes of *S. alterniflora* and the changes indirectly reflected oxidation degree of antioxidant system. Compared without EAN treatment, EAN influenced nitrogen absorption of *S. alterniflora*. During the early stage of growth, *S. alterniflora* only absorbed NH_4^+ through the root tip. As increasing concentration of nitrogen, EAN promoted the absorption of NO_3^- . During the middle period of growth, plant assimilated more NO_3^- than NH_4^+ without nitrogen addition. After EAN was added, *S. alterniflora* absorbed NH_4^+ as only nitrogen source. This means that EAN inhibited the absorption of NO_3^- in the middle of growth. This result was consistent with field crops planted in soil with ammonium nitrogen and ammonium nitrogen inhibited the absorption of NO_3^- (Ramirez et al., 2010; Hawkins and Robbins, 2010). The reason for that was because the EAN was not effectively released during the early stage of growth. *S. alterniflora* preferred to absorb NO_3^- . However, NO_3^- had a slow process of metabolism and synthesis for amino acids and protein (Wakeham et al., 2006). As the plants grow, ammonium nitrogen added in the soil was released, and the plant can directly assimilated NH_4^+ (Balkos et al., 2010). During the last stage of growth, *S. alterniflora* seriously suffered from adverse environment. Researchers found that the plant suffered from poisoning when ammonium nitrogen was the sole source of nitrogen (Feller et al., 2015). The reason that NH_4^+ accumulations were combined action from the conversion process of NO_3^- and NH_4^+ release from the soil. This led to a significant rise in the concentration of NH_4^+ within cells than outside cells (Deak and Malamy, 2005). Concentration difference occurred between the relatively low concentration of ammonium nitrogen outside

cells and the high concentration within cells. Therefore the cells may burst and NH_4^+ efflux increases. The activity of *S. alterniflora* reduced to bottom out.

S. alterniflora played a critical role in ensuring the ecosystem function of coastal wetland. It purified water and regulated the nutrition load of the coastal line (Williamson et al., 2014). Under natural conditions, plants adjusted nitrogen supply for growth through their enzymatic reactions and non-enzymatic reactions (Sinha and Saxena, 2006). The addition of EAN induced self-regulation by inhibiting the *S. alterniflora* root system from absorbing NO_3^- and the absorption of ammonium nitrogen strengthened. The results caused ammonium nitrogen accumulates in rhizosphere environment. At present, coastal eutrophication was expanding and changes of rhizosphere environment led to death of *S. alterniflora* (Deegan et al., 2012). This study also confirmed the influence to plant by nitrogen eutrophication. On the other hand, plant could adjust and control the intension of eutrophication. This study indicated that artificial removal to *S. alterniflora* will restrained the release of nitrogen during the final stage of *S. alterniflora* growth (60 days after the initial growth). The way of plants removed would decrease nitrogen accumulation in the environment. Therefore, wetland plants could reduce the concentration of exogenous nutrition and relieve coastal eutrophication. Restoring wetland plants will be regarded as an important problem in the future researches (Zhao et al., 2014). This research provides a reference and a theoretical basis for restoring coastal wetlands.

Conflict of interest

The authors have no conflict of interest.

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