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Ferrous iron facilitates the formation of iron plaque and enhances the tolerance of *Spartina alterniflora* to artificial sewage stress

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ABSTRACT

The ferrous iron (Fe^{2+}) facilitates the formation of root Fe plaque of wetland plants, but its effect on the tolerance of wetland plants to artificial sewage stress has been seldom reported. In this study, the influences of Fe^{2+} on the formation of Fe plaque and its effects on the tolerance of *Spartina alterniflora* to artificial sewage stress were investigated. The artificial sewage stress decreased the plant height and chlorophyll content and significantly increased the MDA content in leaves. The symptoms of these stresses were alleviated with increasing Fe^{2+} concentration accompanied by significant increase in leaf alcohol dehydrogenase activity. The increase of Fe^{2+} concentration significantly increased the root Fe plaque content and reduced the accumulation of toxic metals in leaves of *S. alterniflora*. These results support our hypothesis that the exogenous Fe^{2+} supply may enhance the stress resistance of *S. alterniflora* to artificial sewage containing heavy metals.

1. Introduction

As a typical salt marsh plant native to Eastern North America, *Spartina alterniflora* L. was introduced to China in 1979 to stabilize coastal banks (Li et al., 2009). The plant has attracted much attention as an invasive plant because of its strong adaptability to climate and competitiveness against native plants (Li et al., 2009; Smith and Lee, 2015). *S. alterniflora* can stabilize and enrich heavy metals and other pollutants; as such, its function in environmental purification has become an important research focus in recent years (Quan et al., 2007; L. Chen et al., 2014; Chen et al., 2017; Negrin et al., 2019). As a major environmental pollutant, heavy metals' accumulation in wetland environment is becoming more and more prominent because of the industrial and municipal waste discharge (Sun et al., 2018). *S. alterniflora* has a strong ability to accumulate heavy metals particularly in in the root (Chen et al., 2017; Negrin et al., 2019). Some heavy metals, including Hg, Cr, Mn, and Pb, can accumulate in considerable amounts in the aboveground parts of *S. alterniflora* (Windham et al., 2003; L. Chen et al., 2014). Besides, as a perennial salt marsh plant, *S. alterniflora* has deep roots and high biomass which is able to tolerate adverse environmental conditions in the coastal wetland (Li et al., 2009). These above characteristics render *S. alterniflora* a potential in the phytoremediation of heavy metal polluted environments, and its function in environmental purification has become an important research focus in recent years (Windham et al., 2003).

A significant feature of wetland plant is the formation of iron (Fe) plaque on roots (Yang et al., 2014; Sebastian and Prasad, 2015; Kaplan et al., 2016; Zandi et al., 2019). Iron oxides or their secondary minerals in Fe plaque have a high specific surface area, which can absorb organic compounds or heavy metals through variable charge surface hydroxyl and various bonding sites, thus playing an important role in adsorption and purification of pollutants (Li et al., 2019; Wei et al., 2019). The two conditions necessary for Fe plaque formation are the large amount of Fe^{2+} in the growth medium and the local oxidization of the rhizosphere environment. The concentration of Fe^{2+} in the medium is one of the important abiotic factors that affect the formation of Fe plaque on the root surface (Li et al., 2016; Tai et al., 2018). A certain range of Fe^{2+} concentration is proportional to the amount of Fe plaque in the roots of wetland plants, such as *Oryza sativa* (Li et al., 2016), *Juncus effusus*, and *Canna indica* (Tai et al., 2018). Li et al. (2016) found that the Fe plaque content in the roots of *O. sativa* increased with increasing Fe^{2+} concentration (20–80 mg L^{-1}). Tai et al. (2018) reported that the Fe plaque levels in *J. effusus* and *C. indica* roots significantly increased to 8.77 and 7.30 g kg^{-1}, respectively, when the Fe^{2+} concentration in the culture solution was increased from 0 mg L^{-1} to 200 mg L^{-1}. When the concentration of Fe^{2+} exceeds a certain range, the Fe plaque content on the root surfaces of *O. sativa* (Fe^{2+} levels exceeding 100 mg L^{-1}) and *Iris pseudacorus* (Fe^{2+} levels exceeding 200 mg L^{-1}) will not increase

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further (Li et al., 2016; Tai et al., 2018). In addition to the coprecipitation and adsorption of heavy metals, some studies have also shown that the presence of Fe plaque contributes to the improvement of plant resistance to abiotic stresses (Sebastian and Prasad, 2016; Fu et al., 2018; Zhang et al., 2019b). Sebastian and Prasad (2016) found that the formation of Fe plaque in the root of *O. sativa* can increase the quantum efficiency of photosynthesis and biomass production when subjected to Cd stress. The formation of Fe plaque in *Camellia sinensis* may also increase the accumulation of nitrogen in the roots and increase the AT-Pase activity in the plasma membrane (Zhang et al., 2019b). Exogenous Fe supplied at a certain range can also accelerate the growth and enhance the tolerance of *Avicennia marina* to Cd stress (Li et al., 2019).

The formation of Fe plaque on the root surface is the result of the oxidation of Fe^{2+} into Fe^{3+} and its precipitation in the form of iron oxides (hydroxides). The redox and migration of Fe are affected by the redox potential (Eh) in the medium and the pH of the rhizosphere, which are in turn influenced by the rate of radial oxygen loss from the plant root (Gambrell et al., 1991; Mei et al., 2012). Radical oxygen loss may help create a rhizosphere oxidation environment and form Eh with different gradients from the root surface to the rhizosphere (Yang et al., 2012). High Eh facilitates the transformation of Fe^{2+} into Fe^{3+} and the formation of iron oxides (hydroxides). Our previous studies have shown that short waterlogging time (corresponding to high soil Eh) contributes to the formation of more Fe plaque on the root of *S. alterniflora* (Zhang et al., 2019a). Low Eh leads to the reduction of Fe^{3+} into Fe^{2+}, releasing bound heavy metals and increasing their bioavailability (Xu and Yu, 2013; Khan et al., 2016). The oxidation of Fe^{2+} also releases H^+, and the formation of Fe plaque is often accompanied by the secretion of organic acids; both phenomena lead to the decrease of rhizosphere pH and affect the availability of heavy metals in sediments (Khan et al., 2016; Sebastian and Prasad, 2016).

Although numerous studies have shown the effect of Fe^{2+} concentration on Fe plaque formation on the root surface, most of these control experiments do not consider the factors of periodic flooding, which will lead to the periodic change of soil Eh and affect the formation of Fe plaque. Besides, the role of Fe plaque in the uptake of nutrients and heavy metals by plants is still controversial. Fe plaque can be either a “reservoir” to the combination and retaining of nutrients, or a “barrier” to the absorption of elements (Zhang et al., 1999; Yang et al., 2014). The transition of this role is believed to be related to the thickness and composition of Fe plaque (Windham et al., 2003; Zandi et al., 2019). The study on the effect of Fe^{2+} on the Fe plaque and its subsequent influences on the absorption of heavy metals by wetland plants is still lacking, especially under the condition of periodic flooding. Moreover, to date, still very few works investigated the effects of Fe plaque formation on the resistance physiology of wetland plants to environmental stresses. In this study, a controlled experiment was conducted in a greenhouse by using *S. alterniflora* as the model plant to evaluate: (1) the formation of Fe plaque and its influences on the migration and transport of heavy metals in *S. alterniflora*; and (2) the effect of Fe plaque formation on the stress response and tolerance of *S. alterniflora* to the artificial synthetic wastewater. We hypothesized that: (1) exogenous Fe^{2+} supply at different concentration has different effect on the formation of Fe plaque and hence the metal uptake in seedlings of *S. alterniflora*; (2) exogenous Fe^{2+} supply may enhance the stress resistance of *S. alterniflora* to artificial sewage containing heavy metals. The results of the present study can provide a theoretical basis for the remediation of heavy metal pollution in the constructed wetland.

2. Materials and methods

2.1. Experimental design

Seedlings of *S. alterniflora* with comparable size (approximately 40 cm in height) were collected from the Nanhui wetland in Shanghai.

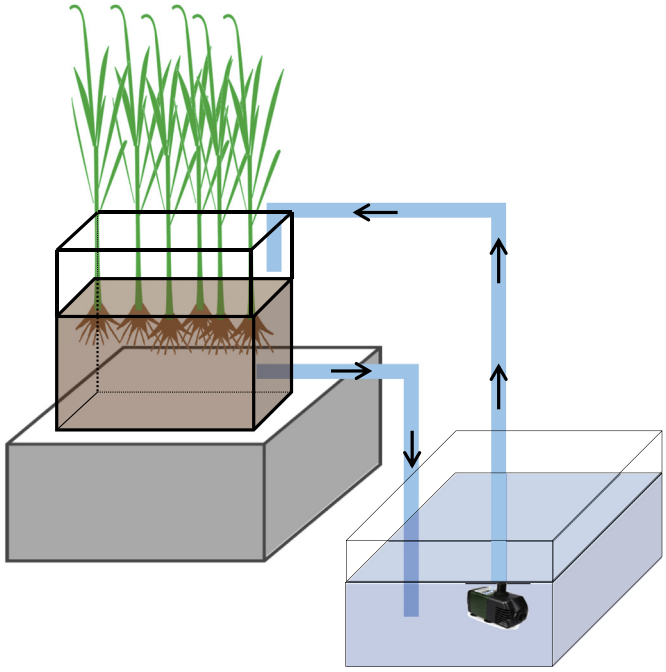

Fig. 1. The schematic diagram of the flooding device.

The collected seedlings were grown in 15 polypropylene pots (32.5 cm [L] × 14 cm [W] × 22.5 cm [H]), with each pot containing 19 kg of quartz sand (0.6–1 mm in particle size) and 1.5 L of full-strength Hoagland solution (Fe was ruled out of the Hoagland solution to preclude its influence on Fe plaque formation). The pots were placed in an artificial climate chamber at a diurnal temperature ranging from 20 °C to 30 °C, a relative humidity of 70% ± 5%, and a light intensity of 800–1400 μmol photons m^{-2} s^{-1} with a 12:12 light:dark photoperiod. An appropriate amount of full-strength Hoagland solution was supplemented every day to compensate for water loss due to evaporation.

After 21 d of rejuvenation, all of the pots were transferred into flooding devices (Fig. 1) were used in our previous study (Zhang et al., 2019a). The pots in the flooding devices were randomly divided into five groups, each in triplicate. The groups were treated with different concentrations of Fe^{2+} (0, 50, 100, 150, and 200 mg L^{-1}) and named Fe0, Fe50, Fe100, Fe150, and Fe200, respectively. Fe^{2+} solution was prepared by dissolving appropriate amounts of $FeSO_4.7H_2O$ in 19 L of full-strength Hoagland nutrient solution for particular levels (50, 100, 150, 200 mg L^{-1}). The Fe^{2+} levels used in the present study were chosen based on a previous study (Liu et al., 2015). All groups were flooded with the treatment solutions in the flooding devices (5 cm above the sand surface) twice a day. All the plants were treated for a week to induce Fe plaque formation in the roots of *S. alterniflora*. After 1 week, the treatment solutions in each flooding device were emptied and replaced with synthetic wastewater (10SW) (Zhang et al., 2019a). The plants were cultured successively for 45 d to determine the effect of Fe plaque formation on the growth, physiology, and metal uptake of *S. alterniflora*. The 1 × SW solution was prepared according to the Shanghai municipal sewage background and Chinese sewage discharge standard by using 0.5 mg L^{-1} NO_3–N, 25 mg L^{-1} $NH4^+$–N, 19.5 mg L^{-1} dissolved organic N, 5 mg L^{-1} PO_4^{3-}–P, 2 mg L^{-1} Cu^{2+}, 5 mg L^{-1} Zn^{2+}, 1 mg L^{-1} Pb^{2+}, and 0.5 mg L^{-1} Cr^{6+}. 10SW treatments were prepared by using 10 times the amount of 1 × SW. All the treatment solutions were renewed every 7 d.

The height of *S. alterniflora* was measured at the end of the experiments. Substrate Eh and pH in the high and low tides of waterlogging were measured by a portable oxidation–reduction potentiometer (Spectrum IQ150, Spectrum Technologies Inc., USA) and a pH meter (FE20, Mettler Toledo, Switzerland). Sampling was performed at

the end of the experiment. One plant selected from each replicate was immediately subjected to radial oxygen loss determination. Another plant in each pot was used for root Fe plaque extraction and porosity measurement. During sampling, the plant was carefully removed from the pot. Residual sand attached to the root was first washed with tap water and rinsed with deionized water triple times. After drying the plant with absorbent paper, a lateral root measuring 8–12 cm was cut into 1 cm pieces; about 0.2 g of the pieces were used for Fe plaque extraction, and another 0.1 g was employed for porosity measurement. The remaining plants were washed. The roots, stems, and leaves were separated, crushed, mixed thoroughly, rapidly frozen in liquid N, and stored at −80 °C until further analyses.

Another hydroponic experiment of *S. alterniflora* was carried out to observe Fe plaque formation and its adsorption on HMs through SEM and energy-dispersive X-ray (EDX) microanalysis. Two *S. alterniflora* plants with comparable sizes were placed into four 1 L beakers containing different concentrations of Fe^{2+} (0, 0, 50, 150 mg L^{-1}). All of the beakers were covered with tinfoil to simulate the root environment in soil. The treatment solutions were prepared using the same methods described above (see the second paragraph of this section). After 7 d, the treatment solution of three groups, that is, 0, 50, 150 mg L^{-1}, were replaced with 20 times the amount of synthetic wastewater (20SW). One treatment without Fe^{2+} (0 mg L^{-1}) received a new Hoagland solution and served as a control. The plants were cultured successively for another 10 d. The lateral root of the plants in each treatment was sampled and fixed with formalin–acetic acid–alcohol for subsequent SEM/EDX visualization.

2.2. Measurement

2.2.1. Root porosity

Root porosity was measured by using the pycnometer method according to Jensen et al. (1969). Lateral roots were rinsed thoroughly with deionized water to remove adhering sands and were cut into approximately 2 cm-long segments. Approximately 0.1 g of fresh roots was weighed in a 25 mL pycnometer. The bottle was filled with distilled water and weighed on an analytical balance (W_{r+w}). The root was removed from the pycnometer, dried with absorbent paper, and immediately weighed on the analytical balance (Wr). The roots were placed into a mortar, ground into a paste, and then returned entirely to the pycnometer. The bottle was topped up with water and weighed again (W_h). The pycnometer filled only with water was also weighted (W_w). All measurements were carried out at room temperature (25 °C). Root porosity (%) was calculated with the following equation: $\text{Porosity}\ (\%) = 100(W_h - W_{r+w})/(W_w + W_r - W_{r+w})$.

2.2.2. Leaf chlorophylls concentration

Leaf chlorophylls concentration was determined according to the method of Chen and Chen (1984). An aliquot of 0.05 g of fresh leaf samples were cut into pieces with stainless steel scissors and then placed in 10 mL of the mixed solution of acetone, ethanol, and distilled water (4.5:4.5:1, v:v). Samples were stored in the dark at 4 °C for 2 d until the leaf tissues whitened. Absorbance readings of the extract were recorded at 645 and 663 nm, and the concentrations (mg L^{-1}) of chlorophyll *a* (Chl a), chlorophyll *b* (Chl b), and total chlorophylls (total Chls) in the extract were calculated in accordance with the empirical formula described by Chen and Chen (1984).

2.2.3. Malonaldehyde

The malonaldehyde concentration in leaves was determined by using the improved thiobarbituric acid method described by Kosugi and Kikugawa (1985). Exactly 0.1 g of fresh leaf sample was homogenized using a mortar and pestle with 5 mL of 10% (w/v) trichloroacetic acid solution. The homogenate was centrifuged at 12,000 rpm for 5 min at 4 °C. Then, 1 mL of supernatant was mixed with an equal volume of 0.6% (w/v) thiobarbituric acid solution. The mixture was kept in boiling water for 30 min and then transferred to an ice bath to stop the reaction. The mixture was then centrifuged at 6000 rpm for 10 min at 25 °C, and the absorbance of the supernatant was measured at 450, 532, and 600 nm. malonaldehyde concentration (C) was calculated by using the following formula: $C = 6.45(A_{532} - A_{600}) - 0.56A_{450}$.

2.2.4. Proline

The determination of proline content was performed according to acid ninhydrin method described by Bates et al. (1973). Approximately 0.1 g of fresh leaf sample ground with 2 mL of 3% sulfosalicylic acid was extracted in a boiling water bath for 10 min. The homogenate was centrifuged at 3000 rpm for 10 min at 4 °C. Then, 1 mL of the supernatant mixed with 1 mL of distilled water, 1 mL of glacial acetic acid, and 2 mL of 2.5% ninhydrin was extracted in a boiling water bath for 1 h and then cooled. The red reaction product in the mixture was extracted with 4 mL of toluene, and the absorbance of red toluene phase was measured at 520 nm using toluene as the blank and L-proline as the standard.

2.2.5. Peroxidase activity

Peroxidase activity was determined by using the guaiacol method (Fielding and Hall, 1978). Approximately 0.1 g of leaf sample was homogenized with 2 mL of ice-cold sodium phosphate buffer (50 mmol L^{-1}, pH 7.4). The homogenate was centrifuged at 12,000 rpm for 5 min at 4 °C. Then 0.1 mL of enzyme extract was mixed with 2 mL of phosphate buffer (50 mmol L^{-1}, pH 7.4) containing 0.2% guaiacol (v/v). The reaction was initiated by adding 1 mL of 0.3% H_2O_2, and guaiacol oxidation was determined based on an increase in the absorbance at 470 nm for 2 min. Per unit enzyme activity of peroxidase (U) is defined as the amount of enzyme that catalyzes the formation of 1 μmol of tetrameric *ortho*-methoxyphenol per minute, and enzyme activity was expressed as U g^{-1} min^{-1}.

2.2.6. Alcohol dehydrogenase activity, anaerobic polypeptides and ethylene content

The leaf and root alcohol dehydrogenase activity and anaerobic polypeptides content, and the root ethylene content were determined by using an enzyme-linked immunosorbent assay kit (Rapidbio, USA) according to instructions of the manufacturer. Approximately 0.1 g of plant sample was homogenized with 1.5 mL 10× phosphate-buffered saline consisting of 137 mmol L^{-1} of NaCl, 2.7 mmol L^{-1} of KCl, 8 mmol L^{-1} of Na_2HPO_4, and 1.46 mmol L^{-1} of KH_2PO_4. Homogenates were centrifuged at 11,000*g* for 5 min at 4 °C, with the supernatant used for determination. The standards of alcohol dehydrogenase, anaerobic polypeptides, ethylene, and samples were added in microplate wells with 100 μL of HRP-conjugate reagent. The microplate was covered with an adhesive strip and incubated for 60 min at 37 °C. A competitive inhibition reaction was started between alcohol dehydrogenase, anaerobic polypeptides, ethylene and HRP-conjugated alcohol dehydrogenase, anaerobic polypeptides, ethylene with the pre-coated antibody specific for alcohol dehydrogenase, anaerobic polypeptides or ethylene. Optical density of the wells was read at 450 nm by using a microtiter plate reader (Infinite M200, Tecan, Switzerland) within 15 min. The detection limit of this assay for alcohol dehydrogenase, anaerobic polypeptides, and ethylene was 0.1 U mL^{-1}, 1 pg mL^{-1}, and 1 pg mL^{-1}, respectively.

2.2.7. Fe plaque content and heavy metal concentration in Fe plaque

Amorphous and crystalline Fe plaque on root surfaces was extracted according to the method by Taylor and Crowder (1983) and Yang et al. (2017). Approximately 0.2 g of *S. alterniflora* root was placed into 25 mL of pre-cooled extract containing 0.2 M of ammonium oxalate (pH 3.2) and 0.14 M of oxalic acid and agitated at 25 °C for 6 h to extract amorphous Fe plaque. For the extraction of crystalline Fe plaque, the roots (after the extraction of amorphous Fe plaque) were rinsed thoroughly with deionized water and then agitated with 25 mL

of extract containing 4/15 M of trisodium citrate ($Na_3C_6H_5O_7{\cdot}2H_2O$) and 1/9 M of sodium bicarbonate ($NaHCO_3$) solution and 3 g of sodium dithionite ($Na_2S_2O_4$) for 15 min. Concentrations of Fe in the two extractants were determined by using inductively coupled plasma–atomic emission spectrometry (ICP-AES, ICAP-7400, Thermo Fisher Inc., USA).

2.2.8. Concentrations of heavy metals in plant tissues and in Fe plaque

The determination of heavy metals in plant parts was performed by using the wet digestion method. Approximately 0.2 g of plant sample was digested in a polyfluortetraethylene beaker with 10 mL of nitric acid, 5 mL of hydrofluoric acid, and 5 mL of perchloric acid. The PTFE beaker was heated on an electric hot plate for 3 h at 200 °C. After digestion, remnants in the beaker were dissolved and washed twice with 5 mL of 1% nitric acid. The washes were combined and mixed with deionized water to yield a final volume of 25 mL. Concentrations of Cu and Zn in the digests were analyzed by using flame atomic absorption spectrometry (AAS, AAnalyst800, Germany). Pb and Cr were determined by using ICP-AES (ICAP-7400, Thermo Fisher Inc. USA). Extracts of amorphous and crystalline Fe plaque were mixed in equal volume and subjected to the same protocol for the analysis of heavy metals. Certified reference material of the carrot (GBW10047) from the China National Center for Standard Materials was used for quality control. The averaged recovery rates (mean and standard deviation) for Cu, Zn, Pb, and Cr in GBW10047 were 96.02 ± 3.69%, 90.19 ± 5.40%, 105.02 ± 13.31%, and 93.06 ± 7.61%, respectively.

2.2.9. Scanning electron microscopy (SEM) and energy-dispersive X-ray (EDX) microanalysis

Selected root samples from the formalin–acetic acid–alcohol fixator was washed with tap water and then rinsed thrice with deionized water. The samples were then subjected to a series of dehydration according to the method by Lu et al. (2014). The dehydrated root samples were subsequently fractured transversely with a sterile knife to obtain a transaction, then coated with gold–palladium, and visualized with SEM–EDX (JSM-6390A, JEOL Ltd., Japan) under 20 kV accelerating voltage and data collection time of 60 s. Fe and heavy metals present on the surface and transverse section of the root were localized by X-ray mapping through EDX analysis.

2.3. Statistical analyses

The mean and standard deviation (SD) of the three replicates for each treatment were calculated. All the data were subjected to a homogeneity test. A parametric one-way analysis of variance (ANOVA) and a Dunnett *t*-test (2-tailed) were conducted to determine significant differences ($p < 0.05$) between the different Fe^{2+} treatment and the control. A one-way multivariate analysis of variance (MANOVA) was conducted to investigate the effects of Fe^{2+} treatment on the metal uptake in roots, leaves and Fe plaque of *S. alterniflora*. If the one-way MANOVA was statistically significant, one-way ANOVAs (the *p* value was subjected to the Bonferroni correction) and Dunnett t-test (2-tailed) tests were further performed. Statistical analyses were performed in SPSS version 16.0.

3. Results

3.1. Changes in substrate Eh and pH and the formation of Fe plaque on S. alterniflora roots

There was a significant main effect for synthetic wastewater and different levels of Fe^{2+} treatment on the Eh of the substrate either under drained ($F(4, 10) = 17.9$, $p < 0.001$) or flooded conditions ($F(4, 10) = 5.2$, $p < 0.012$) as determined by one-way ANOVA, and the substrate Eh increased significantly with increasing Fe^{2+} treatment concentration (Fig. 2). pH showed no notable changes along different levels of Fe^{2+} treatment (Fig. 2). One-way ANOVA also showed significant differences in the crystalline ($F(4, 10) = 25.9$, $p < 0.001$) Fe plaque content along different Fe^{2+} treatment, while the amorphous Fe plaque showed no significant difference ($F(4, 10) = 2.7$, $p = 0.089$). The Fe plaque content in the *S. alterniflora* roots increased with increasing Fe^{2+} concentration, and substantial increases were observed under high levels of Fe^{2+} treatment (Fe150 + 10SW and Fe200 + 10SW) (Fig. 3C). A large amount of amorphous Fe plaque was formed when the treatment Fe^{2+} concentration is low, whereas the content of crystalline Fe plaque increased with increasing Fe^{2+} concentration (Fig. 3C). The reddish-brown color on the root surface of *S. alterniflora* deepened with increasing Fe^{2+} concentration, indicating the increase of Fe plaque content, which was most obvious under high levels of Fe^{2+} treatment (Fe150 and Fe200) (Fig. 3A & B). SEM images showed that obvious deformation and lacunarization of ventilatory tissue cells occurred in the root under the treatment of Fe^{2+} and 20SW (Fig. 4). EDX images showed a high concentration of Fe accumulation on the root surface, especially in the treatment of Fe50 + 20SW and Fe150 + 20SW (Fig. 4).

3.2. Changes of ethylene content and root porosity

There was a significant main effect for different levels of Fe^{2+} treatment on the leaf ethylene content ($F(4, 10) = 18.1$, $p < 0.001$) and root porosity ($F(4, 10) = 19.3$, $p < 0.001$) as determined by one-way ANOVA. Low levels of Fe^{2+} treatments (Fe50 + 10SW and Fe100 + 10SW) significantly increased the root ethylene content (Fig. 5A). The root porosity was also significantly increased by low levels of Fe^{2+} treatment (Fe50 + 10SW) and was considerably higher than other groups (Fig. 5B). With the increasing of Fe^{2+} treatment levels, the root porosity decreased.

3.3. Growth and physiological changes

Different levels of Fe^{2+} treatment showed significant effects on the leaf chlorophyll *a* content ($F(4, 10) = 4.2$, $p = 0.031$), total chlorophylls content ($F(4, 10) = 4.8$, $p = 0.02$), plant height ($F(4, 10) = 5.7$, $p = 0.002$) and leaf malonaldehyde content ($F(4, 10) = 23.2$, $p < 0.001$) according to one-way ANOVA. The chlorophylls content increased with increasing Fe^{2+} concentration, and the significant increases in chlorophyll *a* and total chlorophylls were observed under the treatment of Fe100 + 10SW (Fig. 6A). Different levels of Fe^{2+} treatment also had significant effect on the height of the plant at Day 45 after the treatment. Similar to the responses of chlorophylls, the plant height was also significantly increased under the treatment of Fe100 + 10SW (Fig. 6B). The effect of synthetic wastewater and Fe^{2+} treatment also had significant effect on the leaf malonaldehyde content of *S. alterniflora*. The leaf malonaldehyde content decreased significantly with the increasing of Fe^{2+} concentration (Fig. 6C).

There were significant main effects for Fe^{2+} treatment on the leaf proline content ($F(4, 10) = 28.3$, $p < 0.001$), leaf POD activity ($F(4, 10) = 5.6$, $p = 0.015$), leaf ADH activity ($F(4, 10) = 12.6$, $p = 0.001$) as determined by one-way ANOVA. Treatment of Fe^{2+} showed inhibitory effect on the content of proline and peroxidase activity in leaves of *S. alterniflora*, and high concentration of Fe^{2+} (100 to 200 mg L^{-1}) considerably decreased their content and activity compare to that of the control group (Fig. 7A & B). High concentration of Fe^{2+} treatment (Fe150 + 10SW) significantly increased alcohol dehydrogenase activity in leaves of *S. alterniflora* (Fig. 7C). No significant difference was observed in root ($F(4, 10) = 1.6$, $p = 0.249$) or leaf ($F(4, 10) = 1.2$, $p = 0.378$) anaerobic polypeptides content among different treatments (Fig. 7D).

3.4. Metal accumulation and translocation

According to the one-way MANOVA, there was a statistically

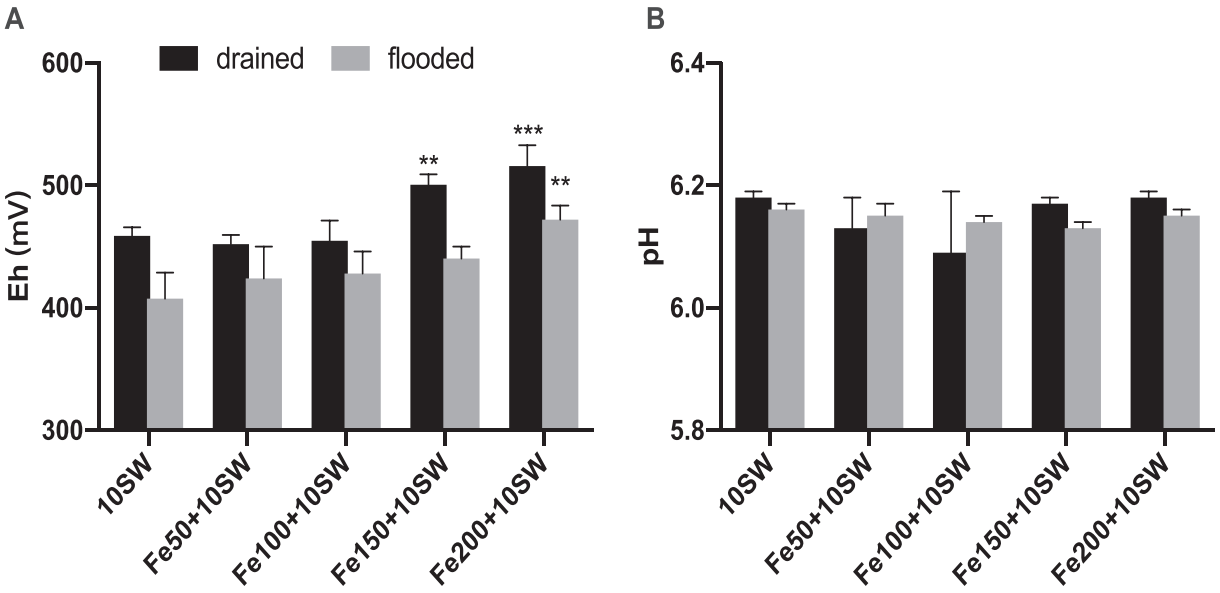

Fig. 2. Changes in substrate (A) Eh and (B) pH under drained and flooded condition along different levels of Fe^{2+} treatment with SW (values are mean and SD; for each parameter, significant difference between treated and control group is indicated by the asterisk, ** and *** indicate significant at $p < 0.01$ and 0.001, respectively).

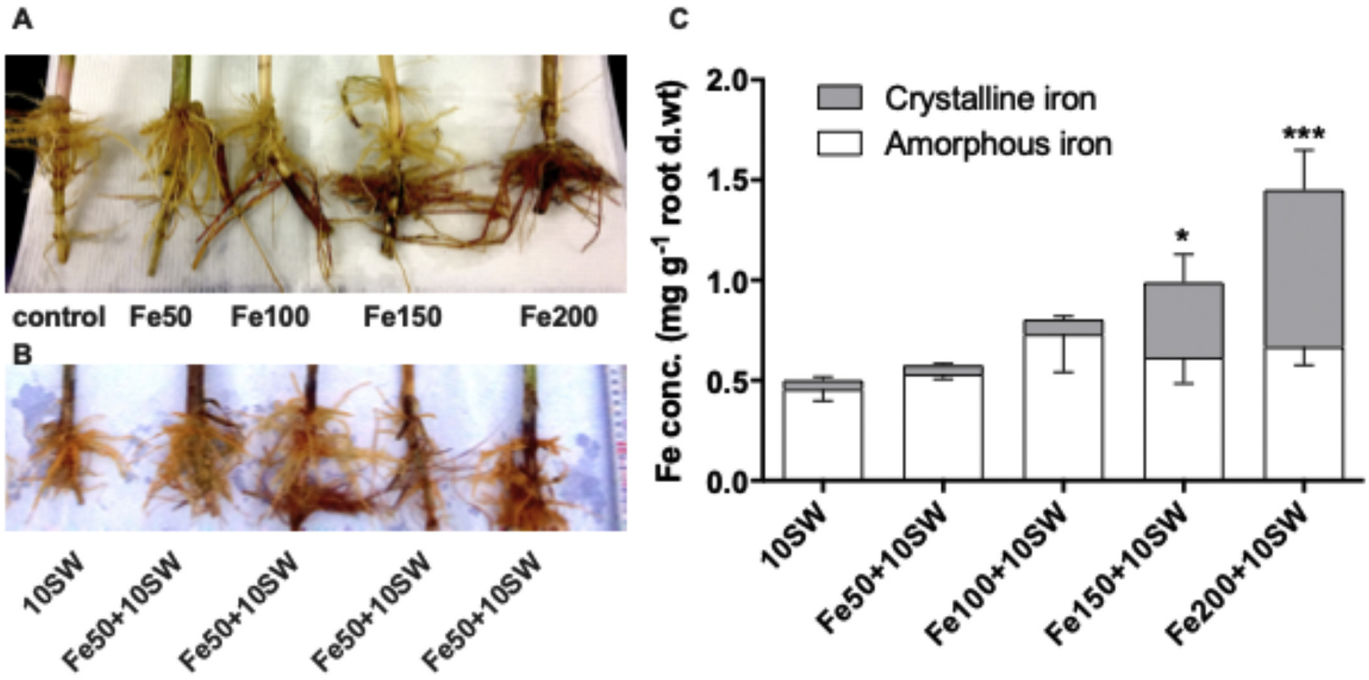

Fig. 3. The appearance of Fe plaque formed on roots of *S. alterniflora* under (A) different levels of Fe^{2+} treatments and (B) different levels of Fe^{2+} treatments with SW; (C) changes in the amorphous and crystalline Fe plaque content on roots of *S. alterniflora* along different levels of Fe2+ treatment with synthetic wastewater (values are mean and SD; for each parameter, significant difference between treated and control group is indicated by the asterisk, * and *** indicate significant at $p < 0.05$ and 0.001, respectively).

significant difference in the metals' accumulation under the treatment of different levels of Fe^{2+} ($F(4, 10) = 18.93$, $p < 0.001$; Wilks' $\Lambda = 0.000$). Further analysis showed that Fe^{2+} treatment had a statistically significant effect on metals' content in roots (Cu, Zn, Pb and Cr), leaves (Cu, Pb and Cr) and the Fe plaque (Cu and Pb) (Table 1). Treatments with 50, 100, and 150 mg L^{-1} Fe^{2+} significantly reduced the content of Cu, Pb, and Cr in leaves of *S. alterniflora*, whereas Fe^{2+} treatment of 50 and 200 mg L^{-1} significantly reduced the accumulation of Zn in *S. alterniflora* leaves (Fig. 8). 50 mg L^{-1} of Fe^{2+} treatments (Fe50 + 10SW) considerably reduced the accumulation of Cu in roots ($p < 0.01$), whereas Cu content in roots treated with high concentration of Fe^{2+} was similar to that in the control (Fig. 8A). Compared with the control treatment (10SW), Fe^{2+} treatment at 150 mg L^{-1} (Fe150 + 10SW) significantly increased the accumulation of Zn in the root of *S. alterniflora* ($p = 0.002$) (Fig. 8B). The accumulation of Pb in the roots of *S. alterniflora* was significantly reduced by Fe^{2+} treatment at various concentrations (Fig. 8C). The accumulation of Cr in the root of *S. alterniflora* was also significantly increased by 150 mg L^{-1} Fe^{2+} treatment (Fe150 + 10SW) compared with 10SW treatment ($p < 0.001$), whereas the Cr content in the root of *S.*

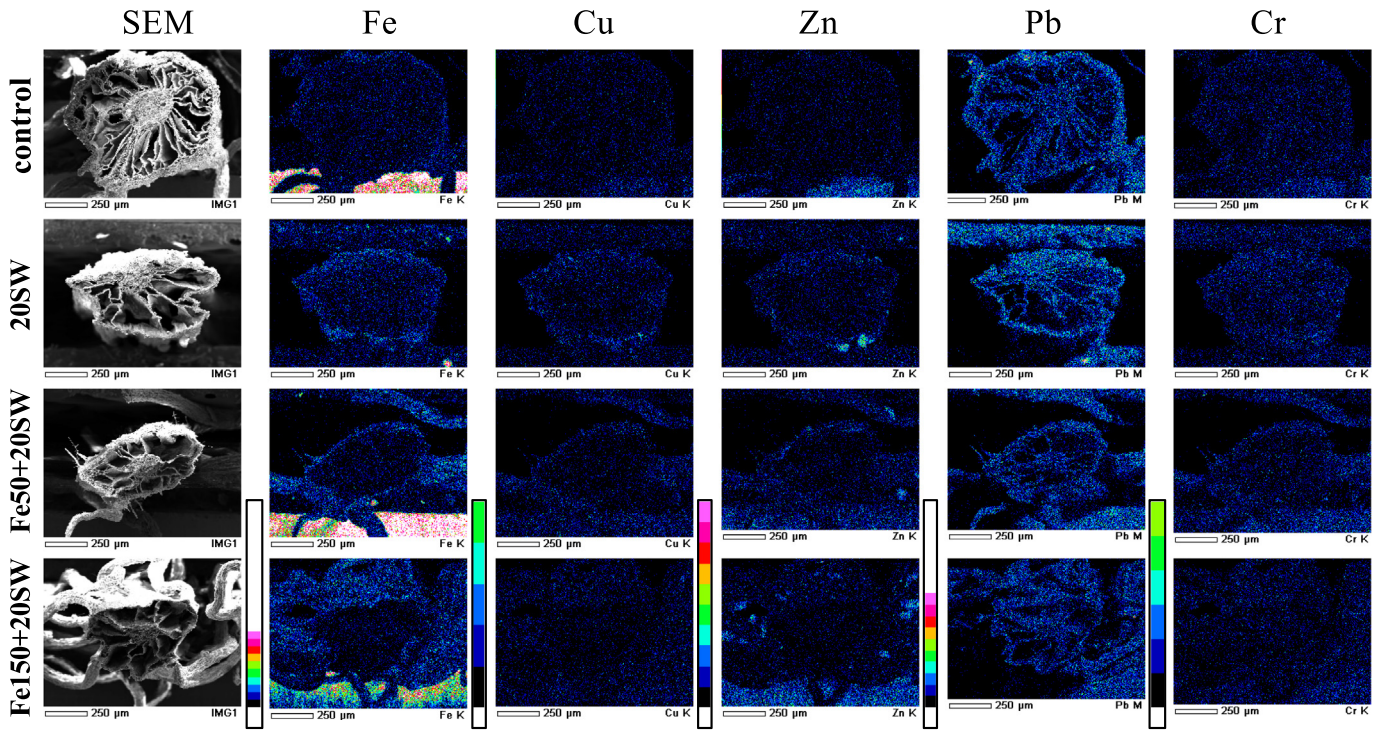

Fig. 4. the SEM observation of the surface (the top row), transection (the middle row) of the root of *S. alterniflora* and the energy dispersive X-ray microanalysis of Fe of the root transection (the bottom row).

alterniflora was significantly decreased by other concentrations of Fe^{2+} treatment (Fig. 8D). Fe^{2+} treatment had no significant effect on the translocation factor of Cu, whereas the translocation factor of Zn showed a downward trend with increasing Fe^{2+} concentration, high levels of Fe^{2+} (Fe150 + 10SW and Fe200 + 10SW) significantly decreased the translocation of Zn to the aboveground part of *S. alterniflora* (Table 2). The translocation factor of Pb was considerably increased by the high concentration of Fe^{2+} treatment (Fe200 + 10SW), whereas that of Cr was significantly decreased by 50 to 200 mg L^{-1} of Fe^{2+} treatment (Fe50 + 10SW, Fe150 + 10SW and Fe200 + 10SW) compared with the 10SW treatment ($p = 0.004$) (Table 2).

4. Discussion

Wetland plants treated with Fe^{2+} usually induce the precipitation of iron oxides and hydroxides and turns root surfaces into a deep reddish-brown color (Siqueira-Silva et al., 2012). In this study, the roots of *S. alterniflora* turned reddish-brown one week after the treatment of Fe^{2+} (Fig. 3). This result indicates Fe plaque formation on root surfaces. The color deepened with increasing Fe^{2+} concentration, and the root apex was even blackened by the high level of Fe^{2+}, which may be induced by the increased accumulation of iron oxides and hydroxides on the root of *S. alterniflora*. Quantitative determination of DCB extraction and electron microscopy confirmed that the content of Fe plaque on the root

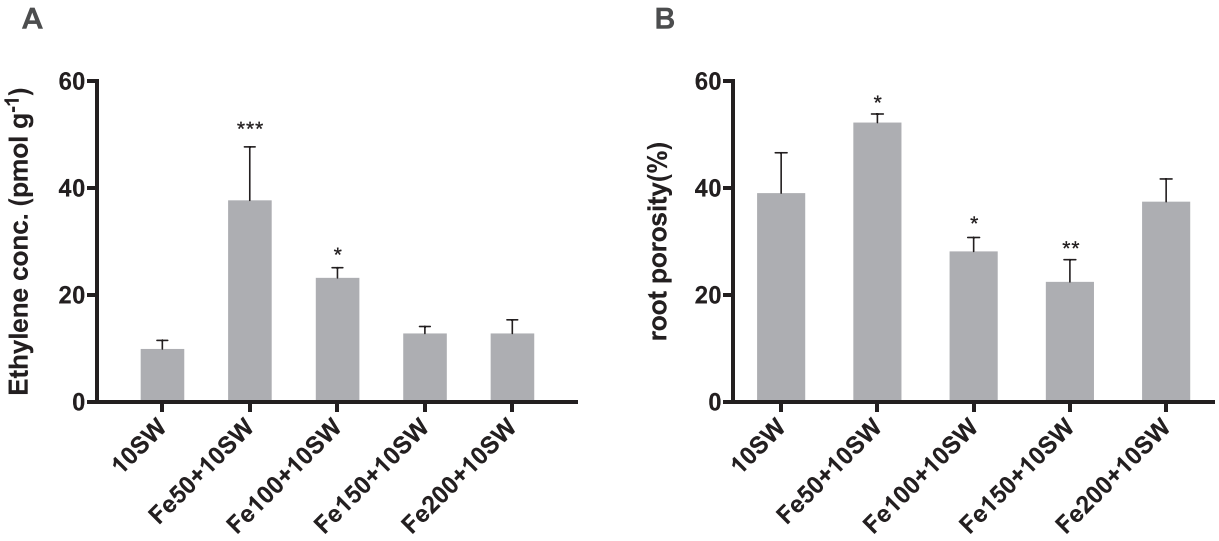

Fig. 5. Changes of (A) ethylene content and (B) root porosity of *S. alterniflora* under different levels of Fe^{2+} treatments with SW (values are mean and SD; for each parameter, significant difference between treated and control group is indicated by the asterisk, *, ** and *** indicate significant at $p < 0.05$, 0.01 and 0.001, respectively).

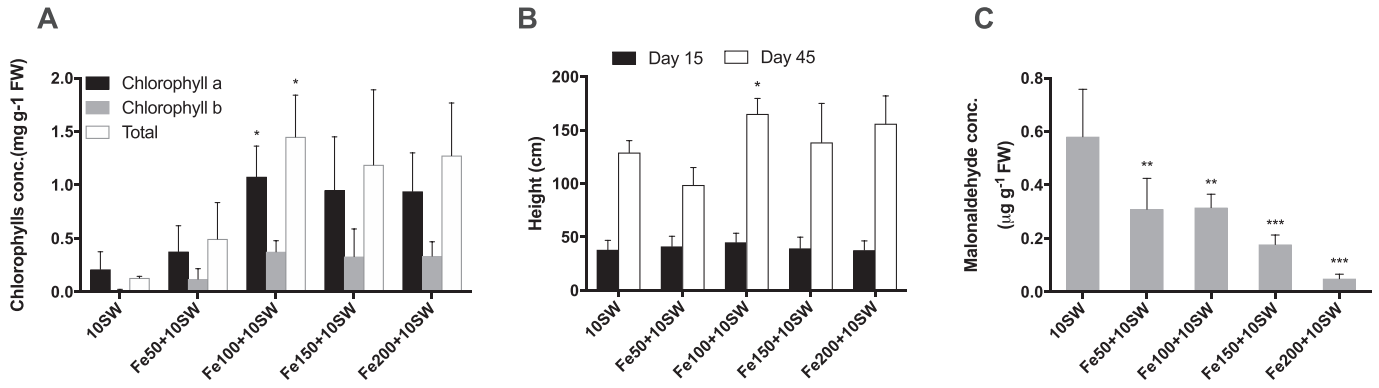

Fig. 6. Changes of (A) leaf chlorophylls content, (B) plant height and (C) leaf MDA content of *S. alterniflora* under different levels of Fe^{2+} treatments with SW (values are mean and SD; for each parameter, significant difference between treated and control group is indicated by the asterisk, *, ** and *** indicate significant at $p < 0.05$, 0.01 and 0.001, respectively).

surface of *S. alterniflora* increased with increasing Fe^{2+} concentration (Fig. 3). This result is consistent with many previous studies. Increasing Fe^{2+} concentration in the substrate can considerably increase Fe plaque content on root surfaces of *O. sativa* (Syu et al., 2013; M. Chen et al., 2014) and *Glyceria spiculosa* (Fr. Schmidt.) Rosh (Jia et al., 2018). In the present study, the composition of Fe plaque on roots of *S. alterniflora* varies with Fe^{2+} concentration. Amorphous Fe plaque was dominant when the treatment Fe^{2+} concentration was low, and crystalline Fe plaque increased significantly with increasing Fe^{2+} concentration. The results are consistent with previous studies that the amorphous form of Fe plaque is dominant on the root surface of wetland plants (Weiss et al., 2004). In the process of Fe plaque formation, different forms of Fe plaque can be transformed into one another, and this transformation will be affected by the medium Eh and the concentration of Fe^{2+} (Liu

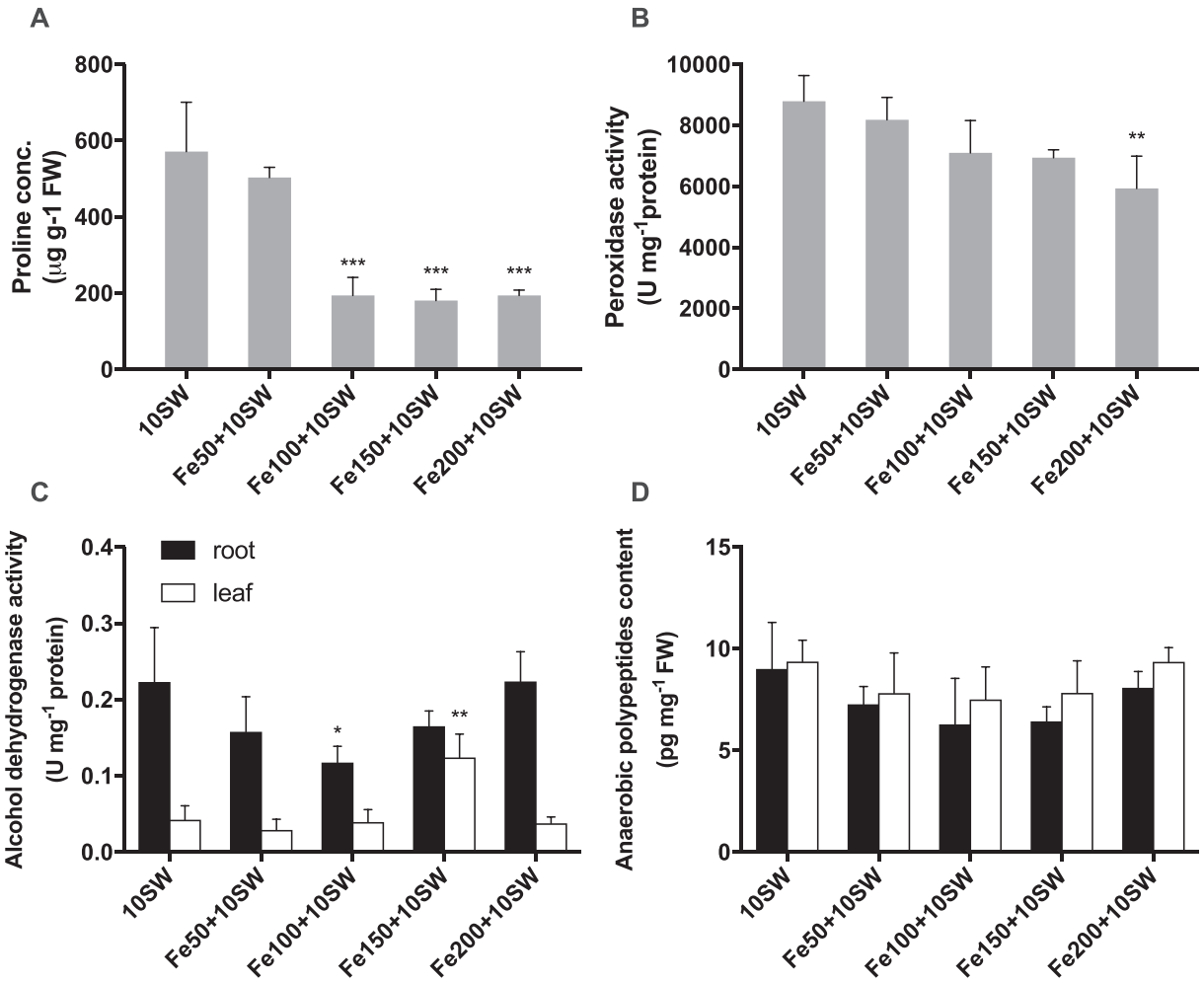

Fig. 7. Changes of (A) proline content, (B) POD activity, (C) ADH activity and (D) ANPs content in leaf of *S. alterniflora* under different levels of Fe^{2+} treatments with SW (values are mean and SD; for each parameter, significant difference between treated and control group is indicated by the asterisk, *, ** and *** indicate significant at $p < 0.05$, 0.01 and 0.001, respectively).

Table 1
One-way MANOVA results showing the effects of different levels of Fe^{2+} on different sources of variations (Degrees of freedom for the Fe^{2+} treatment is 4; MS, the between groups mean sum of squares; *p*, probability).

Sources of variations	MS	F	*p*
Root Cu content	19,938.817	6.847	0.007
Leaf Cu content	439.803	6.334	0.008
Fe plaque Cu content	9284.728	4.960	0.018
Root Zn content	133,511.696	19.847	< 0.001
Leaf Zn content	6588.899	3.091	0.067
Fe plaque Zn content	918.155	0.265	0.894
Root Pb content	159.937	12.317	0.001
Leaf Pb content	62.864	56.951	< 0.001
Fe plaque Pb content	24.442	4.127	0.031
Root Cr content	186.677	137.784	< 0.001
Leaf Cr content	44.618	96.859	< 0.001
Fe plaque Cr content	4.507	0.317	0.860

et al., 2005; Li et al., 2020). Changes in medium Eh may favor the process of ferrolysis and thus influence the crystallinity of iron oxide (Li et al., 2020). Fe^{2+} is considered to be a catalyst in the phase transformation of iron plaque, because Fe^{2+} can catalyze the transformation of ferrihydrite into stable crystalline iron oxides through dissolution/reprecipitation mechanism, and transform ferrihydrite into hematite directly by dehydration and rearrangement (Liu et al., 2005). In the present study, high levels of Fe^{2+} treatment likely catalyzed the transformation of amorphous Fe plaque into crystalline forms through the above mechanisms, which lead to the increase of crystallinity of iron oxides in the Fe plaque on roots of *S. alterniflora*.

The formation of Fe plaque can promote or inhibit the growth and physiology of plants, and the effect and degree may vary with the content of the Fe plaque and environmental conditions (Greipsson, 1994; Fu et al., 2018; Zhang et al., 2019b). With the increase of Fe^{2+} concentration, the content of Fe plaque in the root increased significantly, along with the increase of plant height and the decrease of malondialdehyde and proline content in the leaves. Malondialdehyde is an important indicator of lipid peroxidation caused by cell oxidative stress, and the proline level of plant tissue is generally proportional to the degree of stress (Schat et al., 1997; Lu et al., 2014). These results indicate that the increase of Fe plaque content can help reduce membrane peroxidation and alleviate the peroxidation damage of *S. alterniflora*. In addition to its possible role in interfering with the absorption of harmful metals, the formation of the Fe plaque is also believed to consume the active oxidants secreted by roots and thus play a role in plant antioxidant stress (Fu et al., 2018). A certain dose of ferrous iron has been proved to be a regulator for increasing amino acid content and enzyme activity of plants (Fu et al., 2018; Zhao et al., 2019). A previous study also showed that the deposition of Fe plaque on roots of plant can

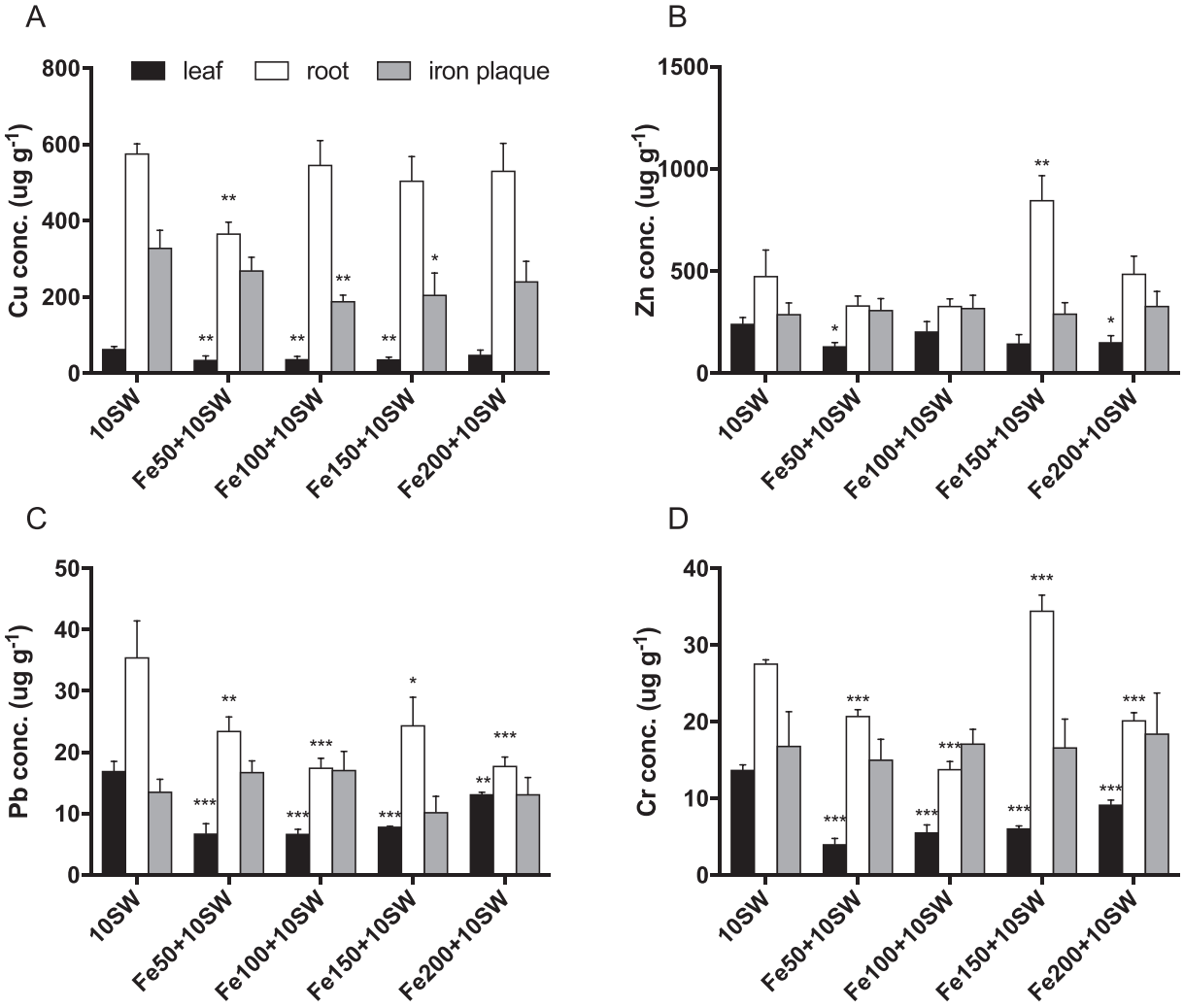

Fig. 8. The concentration of (A) Cu, (B) Zn, (C) Pb and (D) Cr in the root, leaf and DCB extract of *S. alterniflora* under different levels of Fe^{2+} treatments with SW (values are mean and SD; for each parameter, significant difference between treated and control group is indicated by the asterisk, *, ** and *** indicate significant at $p < 0.05$, 0.01 and 0.001, respectively).

Table 2
Translocation factors (TF) of Cu, Zn, Pb and Cr in *S. alterniflora* under different levels of Fe^{2+} with SW. (The degree of freedom for the treatment is 4; values are mean and SD; for each metal.)

TF	10SW	Fe50 + 10SW	Fe100 + 10SW	Fe150 + 10SW	Fe200 + 10SW
Cu	0.11 ± 0.01	0.10 ± 0.03	0.07 ± 0.01	0.07 ± 0.00	0.09 ± 0.02
Zn	0.53 ± 0.14	0.40 ± 0.01	0.51 ± 0.20	0.21 ± 0.04*	0.26 ± 0.08*
Pb	0.48 ± 0.05	0.29 ± 0.10*	0.39 ± 0.05	0.36 ± 0.06	0.74 ± 0.07**
Cr	0.50 ± 0.03	0.20 ± 0.04***	0.40 ± 0.08*	0.18 ± 0.01***	0.46 ± 0.01

* Difference between treated and control group is significant at $p < 0.05$.
** Difference between treated and control group is significant at $p < 0.01$.
*** Difference between treated and control group is significant at $p < 0.001$.

help to alleviate the toxic effects of heavy metals, and plants with more Fe plaque had significantly better plant growth including the greater dry weight of roots and leaves (Greipsson, 1994). When Fe^{2+} concentration is low, this alleviation effect seems to disappear. According to the results of this study, the plant height, the leaf chlorophyll and the malondialdehyde content of *S. alterniflora* treated with low concentration Fe^{2+} (Fe50 + 10SW) are not significantly different from the 10SW treatment (Fig. 6). The root porosity and ethylene content of *S. alterniflora* roots both reached the maximum level under the low Fe^{2+} treatment. The root porosity and the increase of ethylene content are the result of the aging and differentiation of root cells (Yamauchi et al., 2018; Munir et al., 2019). The treatment with low Fe^{2+} concentration seems to accelerate the aging and differentiation of root cells, and its internal mechanism needs further study.

In the present study, the activity of peroxidase and anaerobic polypeptides did not significantly change with increasing Fe concentration and some of these antioxidants even decreased (e.g. peroxidase), while the leaf alcohol dehydrogenase activity significantly by the Fe^{2+} treatment at 150 mg L^{-1}. These results suggest that the mitigation of peroxidation damage of *S. alterniflora* membranes from Fe plaque formation has little relationship with the response of peroxidase and anaerobic polypeptides but may have a correlation with alcohol dehydrogenase activity. We have not measured the activities of other antioxidative enzymes, such as superoxide dismutase, peroxidase and catalase, in the leaves of *S. alterniflora*. However, according to previous studies, the formation of Fe plaque with the participation of Fe^{2+} can help plants improve the activities of these enzymes in rice subjected to Cd stress (Fu et al., 2018). Another study by Zhang et al., 2019b also found that efficient iron plaque formation is linked to increased activities of root enzymes such as plasma membrane H^+-ATPase. These results indicate that the regulatory effect of Fe plaque formation on antioxidant enzymes in different plants varies with plants and the types of antioxidant enzymes.

The formation of Fe plaque on the root surface of wetland plants can promote or inhibit the absorption of heavy metals by plants. This effect is not only related to the thickness of Fe plaque but also depends on the types of heavy metals (Liu et al., 2007; Feng et al., 2013). In this study, although Fe plaque content increased significantly with the addition of Fe^{2+}, the concentration of heavy metals in the Fe plaque did not considerably increase compared with the 10SW group, which indicated that Fe plaque can adsorb certain heavy metals, but the concentration of heavy metals adsorbed by the Fe plaque did not increase with increasing Fe plaque content. This result may be due to the decrease of the binding sites with heavy metals due to the increase of Fe plaque thickness (Liu et al., 1999). When the concentration of Fe^{2+} in the medium was 150 mg L^{-1}, the absorption of Zn and Cr in the root of *S. alterniflora* increased accompanied by significantly increased crystalline Fe plaque content. This result may be partly attributed to the fact that the specific surface area of crystalline Fe plaque is smaller than that of amorphous Fe plaque, and its adsorption and retention effect for heavy metals is weaker than that of amorphous Fe plaque, which results in excessive intake of these heavy metals in roots. However, it should be noted that the effects of different amounts of Fe plaque on the absorption of heavy metals in plant roots vary with the types of heavy metals. Compared with Zn and Cr, the increase of Fe plaque content did not significantly affect the accumulation of Cu and Pb in the root of *S. alterniflora*.

Compared with the 10SW group, the accumulation of Cu, Zn, Pb, and Cr in the leaves of *S. alterniflora* was reduced considerably by Fe^{2+} treatments, and increased Fe plaque content on root surfaces seemed to impede the translocation of metals to aboveground parts of plants. Our results are similar to those of Xu and Yu (2013) and Xu et al. (2018); when Fe plaque forms on the root surface of *O. sativa*, Zn and Cr contents in leaves were significantly lower than those without Fe plaque. Fu et al. (2018) also found that adding 0.2 mmol L^{-1} of Fe^{2+} to the culture solution can considerably reduce the uptake of Cd in the aerial part of *O. sativa*. As a divalent cation, Fe^{2+} can compete with other divalent cations for binding sites, thus affecting the absorption and translocation of other heavy metals (Fu et al., 2018). Although the absolute amount of heavy metals accumulated in leaves of *S. alterniflora* decreased with the increase of Fe plaque content, the relative allocation of heavy metals in roots and leaves (reflected as translocation factor) showed different characteristics. The translocation factor of Cu and Zn generally decreased with the increase of Fe plaque content. The translocation factor of Pb and Cr, however, only decreased when the Fe plaque content was low. Under the high concentration of Fe^{2+} treatment (Fe200 + 10SW), the translocation of Pb in the aerial part of *S. alterniflora* was significantly increased compared to the control treatment (10SW) (Table 2), and the concentration of Pb and Cr in the aerial parts of *S. alterniflora* also increased with increasing Fe^{2+} concentration (Fig. 8). These results indicated that a high concentration of Fe^{2+} on the root surface of *S. alterniflora* not only enhanced the tolerance to wastewater stress but also helped to transport Pb and Cr to the aboveground parts of plants. These characteristics implicate that *S. alterniflora* has the potential to be used as a phytoremediation species to remediate wastewater borne Pb and Cr pollution in the coastal environment. Mei et al. (2014) investigated the absorption of heavy metals in artificial wastewater by wetland plants and found that plants with Fe plaque had stronger tolerance to heavy metals and higher removal capacity of heavy metals than those without Fe plaque. The existence of Fe plaque plays a certain role in the accumulation and translocation of heavy metals in *S. alterniflora*, but the root of the plant is the major pool for heavy metals, and the translocation of most heavy metals to the aerial part is limited. These findings indicate that the root system is the main barrier affecting the translocation of heavy metals in *S. alterniflora*. Our results are consistent with many previous studies, that the roots system has proven to be a major obstacle to Cd translocation in the aerial parts of mangroves (*A. marina* and *Kandelia obovata*) (Dai et al., 2017) and rice (*O. sativa*) (Liu et al., 2007).

5. Conclusions

This study revealed the dose-dependent positive correlation between the root Fe plaque content of *S. alterniflora* and exogenous Fe^{2+} concentration. Our results support the hypothesis that the increases in the Fe plaque content has a protective effect on *S. alterniflora* seedlings

under the stress of artificial sewage containing heavy metals. This protective effect is mainly through reducing the translocation of toxic metals to the aboveground sensitive parts (leaves) and improving the activity of antioxidant enzymes (such as alcohol dehydrogenase), so as to reduce the oxidative stress and promote the plant growth. In addition to confirming the previous studies that the formation of Fe plaque is promoted by ferrous iron, this study further revealed that different concentrations of ferrous iron also have a significant impact on the composition of Fe plaque. High concentrations of ferrous iron lead to a significant increase in the content of crystalline Fe plaque, and the influence of different composition of Fe plaque on the absorption of heavy metals and the plant resistance needs to be studied further. It must be noted that the actual role of the Fe plaque is closely related to its quantity on the root surface. Excessive assimilation of Fe in root as Fe plaque may have a deleterious effect on the growth and physiology of the wetland plants. The excessive Fe plaque may interfere with the uptake of micronutrients and in the long run eliminate the plants from the Fe polluted environments (Siqueira-Silva et al., 2012). Further ecotoxicological studies are called to assess the effectiveness of Fe^{2+} addition on the in-situ remediation of *S. alterniflora* on wastewater-borne heavy metals in field conditions.

CRediT authorship contribution statement

Qiqiong Zhang: Writing - original draft, Investigation, Resources, Formal analysis. **Zhongzheng Yan:** Conceptualization, Writing - original draft, Supervision, Writing - review & editing. **Xiuzhen Li:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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