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Physiological responses of three plant species exposed to excess ammonia in constructed wetland

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ABSTRACT

Constructed wetlands were widely used for the treatment of effluents rich in ammonia, but wetland plants might be affected under high ammonia concentration. Experiments were conducted to assess the effect of increased ammonia concentration on wetland plants, by examining the changes of chlorophyll content and antioxidant systems in *Typha angustifolia, Scirpus validus* and *Zizania latifolia*. Results showed that ammonia levels in excess of 100 mg·L^{-1} significantly reduced total chlorophyll content for *T. angustifolia*, and levels up to 400 mg·L^{-1} similarly reduced total chlorophyll content for *S. validus*. No significant decrease of total chlorophyll content for *Z. latifolia* were observed. When ammonia levels were up to 100, 200 and 300 mg·L^{-1}, it could generate oxidative stress in *T. angustifolia, S. validus and Z. latifolia respectively, expressed through an elevated malondialdehyde (MDA) content and the enhancement of superoxide dismutase (SOD), catalase (CAT), peroxidase (POD) activities. It was suggested that excess ammonia in wastewater could affect the physiological responses of wetland plants, by inhibiting photosynthesis and inducing oxidative stress. And* Z. latifolia showed a higher tolerance to ammonia than *T. angustifolia* and *S. validus*.

Keywords: Constructed wetland; *Typha angustifolia*; *Scirpus validus*; *Zizania latifolia*; Ammonia tolerance

1. Introduction

Over the last decades, constructed wetlands have been widely used to treat a wide variety of wastewaters, especially municipal, agricultural, animal wastewater and landfill leachate [1,2]. Compared with the conventional wastewater treatment system, it has low construction and operational costs [3]. But high pollutant concentrations in wastewater might destroy the constructed wetland system, because the adverse conditions may affect wetland plants.

Ammonia is a major pollutant in wastewater treated by constructed wetlands, and the concentrations often exceed 100 mg·L^{-1} and reach as high as 400 mg·L^{-1} [4–6]. Concentrations in this range may exceed plant needs, and inhibit plant growth. It has been shown that a high concentration of ammonia is highly toxic to plants [7]. Excess ammonia caused reduction in photosynthesis and nutrient uptake of plants [8]. As a result, plants show various symptoms of injury, such as chlorosis, growth inhibition, and finally death. Additionally, excess ammonia could trigger oxidative stress in plants by accumulating reactive oxygen species (ROS) which could be scavenged by antioxidative enzymes [9]. The main antioxidative enzymes

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in plants are superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT). Oxidative stress can cause damage to nucleic acids and proteins and is one of the main causes for productivity decreases, injury, and death in plants. Therefore, chlorophyll content and the activity of antioxidative enzymes were always used as physiological indicators of ammonia stress in plants [8–14].

It has been accepted that there are great differences in ammonia tolerance among wetland species. The growth of *Typha latifolia* L., *Juncus effuses* L. and *Schoenoplectus tabernaemontani* were found to be inhibited by ammonia concentrations in the range of 150–350 mg·L^{-1}, and T*ypha angustifolia* was not significantly affected in ammonia concentrations below 400 mg·L^{-1} [15,16]. However, little work has been done to study the relationship between ammonia tolerance and physiological responses in wetland plants. A better understanding of the physiological and biochemical mechanism conferring ammonia tolerance is very important.

In light of the above, the present study was conducted on three emergent aquatic macrophytes that are widely used in constructed wetlands, *Typha angustifolia*, *Zizania latifolia* and *Scirpus validus*. Changes of total chlorophyll content, lipid peroxidation and antioxidative enzymes in the three wetland plants were studied to better understand their susceptibility to ammonia stress. And the results could provide useful data for the ammoniated effluent treatment through constructed wetlands.

2. Materials and methods

2.1. Plant material

Three wetland plants *T. angustifolia*, *Z. latifolia* and *S. validus* were collected from the constructed wetlands in Nansihu Lake in China, and then planted in plastic barrel (28 cm in height, 30 cm in diameter) filled with 15 cm clean river sand and 10 L water. Each barrel contained 4 plants for *T. angustifolia*, 5 plants for *Z. latifolia* and 8 plants for *S. validus*. Before the experiment, plants were cultivated outdoors for at least 4 weeks in 10% Hoagland solution [17].

2.2. Experimental design

The experiment was conducted outdoors under a transparent roof in the Baihua Park (36°40′36″ N, 117°03′42″ E), Jinan, China, in the summer of 2009. The plants were treated with 0 (control), 50, 100, 200, 300 and 400 mg·L^{-1} $(NH_4)_2SO_4$ for thirty days while growing in 10% Hoagland solution. Each treatment was conducted in duplicate. During the experiment, the culture was exchanged every two days and the pH of the solution was adjusted to 7.0 (± 0.2) every day using HCl/NaOH. At the end of the experiment, the plant leaves in each aquarium were harvested and divided into two parts, then immediately stored in liquid nitrogen for the analysis of protein, malondialdehyde (MDA) content, and antioxidative enzymes activities.

2.3. Determination of photosynthetic pigments

The plant leaves were grounded in liquid nitrogen, and then extracted with 25 mL 80% acetone in darkness, at 4°C for 24 h. The contents of chlorophyll a and b were determined as described in Lichtenthaler [18].

2.4. Extraction of enzymes

Plant samples (about 0.5 g) were grounded in liquid nitrogen using mortars and pestles, and the ground samples were homogenized with 5 mL pre-chilled phosphate buffer (0.05 M, pH = 7.0) containing 1% polyvinylpyrrolidone and 1 mM EDTA. The homogenates were centrifuged at 12,000 × g for 15 min at 4°C, and the supernatants were used for enzyme assays and protein content.

2.5. Enzyme activity measurement and protein determination

SOD activity was assayed based on the method of Beauchamp and Fridovich, which measured the inhibition of nitro-blue tetrazolium (NBT) photochemical reduction at 560 nm [19]. One unit SOD activity was defined as the amount of enzyme required to inhibit 50% NBT reduction rate. CAT activity was determined by the measurement of the H_2O_2 decomposition at 240 nm, according to method of Aebi [20]. One unit CAT activity was defined as the amount of enzyme required to decompose 1 nmol H_2O_2 per min. POD activity was determined as oxidation of guaiacol by H_2O_2, according to Kraus and Fletcher [21]. One POD unit was defined as the amount of enzyme required to decompose 1 μmol guaiacol per min. SOD, POD and CAT activities were expressed as SI unit activity per mg of protein.

Protein in the supernatant was assayed by the Coomassie brilliant blue-dye binding method of Bradford [22]. The results were expressed as mg of protein per g fresh weight of leaves.

2.6. Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 0.5 g leaf fresh weight according to Heath and Packer [23]. MDA is a product of lipid peroxidation by thiobarbituric acid reaction. The MDA content was expressed as nmol per g fresh weight of leaves.

2.7. Statistical analysis

Each date point in the mean of two replicates obtained from two independent experiments (n = 4). The mean differences were compared by t-test using the statistical software package for social science (SPSS) version 11.0,

and differences were considered to be significant at the level of $P < 0.05$.

3. Results

3.1. Effect of ammonia on total chlorophyll content

Total chlorophyll content of *T. angustifolia, Z. latifolia* and *S. validus* is shown in Fig. 1. The chlorophyll content of three species showed great differences with ammonia treatments. Compared with control groups, decrease in total chlorophyll content of *T. angustifolia* at 50 mg·L^{-1} ammonia concentration was not significant but was remarkable above this concentration. A significant increase was observed at 50, 100, 200 and 300 mg·L^{-1} ammonia concentrations for *Z. latifolia*. And a great increase in *S. validus* was also detected at concentrations of 50, 100 and 200 mg·L^{-1} ammonia, but a significant decrease was observed at 400 mg·L^{-1} ammonia treatments, compared with controls (Fig. 1).

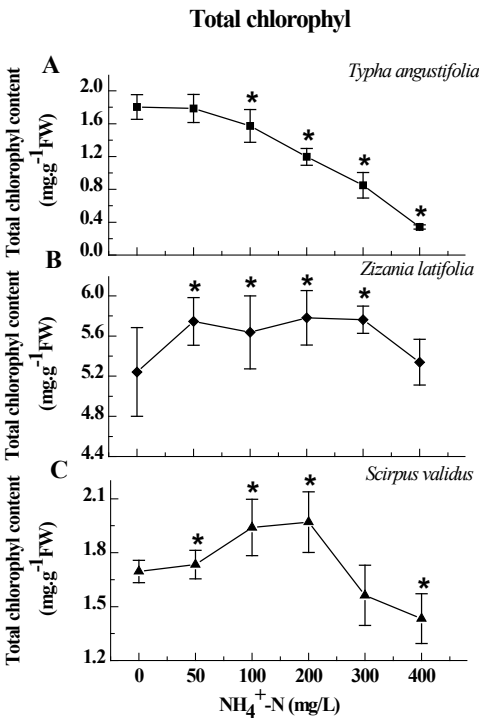

Fig. 1. Effects of different concentrations of ammonia on total chlorophyll contents in (A) *T. angustifolia*, (B) *Z. latifolia* and (C) *S. validus*. Values are mean ± S.E (standard error). Asterisks (*) indicate that mean values are significantly different between ammonia treatments and controls ($n = 4$, $P < 0.05$).

3.2. Effect of ammonia on antioxidative enzyme activity

SOD activity of three wetland plants under the effect of different ammonia concentrations is shown in Fig. 2. Exposure of *T. angustifolia* to 100, 200 and 300 mg·L^{-1} ammonia caused a general increase of SOD activity. And a significant increase was observed at ammonia concentrations of 300 mg·L^{-1} for *Z. latifolia*, and 200, 300 mg·L^{-1} for *S. validus*. Whereas, at the highest concentration of ammonia, which was 400 mg·L^{-1}, SOD activity decreased to the levels of controls in the three species.

POD activity, which decomposes the H_2O_2, was significantly different among the three test species. When *T. angustifolia* was exposed to 100, 200, 300 and 400 mg·L^{-1}, and *Z. latifoli*a to 400 mg·L^{-1} of ammonia, the POD activities were significantly increased, compared to controls. However, unlike the other two species, the POD activity of *S. validus* did not increase significantly, and the activity levels were very low (Fig. 2).

CAT activity, another scavenger of H_2O_2, in *T. angustifolia, Z. latifolia* and *S. validus* is shown in Fig. 2. For *T. angustifolia*, CAT activity was greatly larger than the controls when treated with 100, 200 mg·L^{-1} of ammonia, and a significant decrease was observed at ammonia concentrations of 400 mg·L^{-1}. Unlike *T. angustifolia*, CAT activity in *Z. latifolia* increased significantly, when treated with 300–400 mg·L^{-1} ammonia. For *S. validus*, the CAT activity was also increased significantly at 200 and 300 mg·L^{-1} ammonia treatments; but like *T. angustifolia*, the CAT activity was also lower than controls under 400 mg·L^{-1} ammonia.

3.3. Effect of ammonia on lipid peroxidation

Membrane lipid peroxidation in the leaves of three wetland species was assessed as the content of MDA. The effect of ammonia stress on MDA formation in the leaves of three species after 30 day treatment is shown in Fig. 3. MDA content of *Z. latifolia* has not been greatly increased from ammonia concentrations at the studied concentration range (50, 100, 200, 300, 400 mg·L^{-1}) compared to the controls. And when ammonia levels were in excess of 200 and 400 mg·L^{-1}, it could significantly raise the MDA contents of *T. angustifoli*a and *Z. latifolia* separately. Therefore, ammnia-induced increase in lipid peroxidation was significantly higher in *T. angustifolia* than that in other two species.

4. Discussion

It has been demonstrated that ammonia stress can inhibit growth, decrease photosynthesis efficiency and induce oxidative stress in plant tissues [7]. However, most of reports focused on terrestrial plant or submersed plant, while few studies on the effects of ammonia stress to wetland macrophyte, though constructed wetlands were widely used to treat ammoniated effluent [13,15]. Study of the chlorophyll content and antioxidant responses of *T. angustifolia, Z. latifolia* and *S. validus* to ammonia stress is a good approach to better understanding the tolerance capacity of plants.

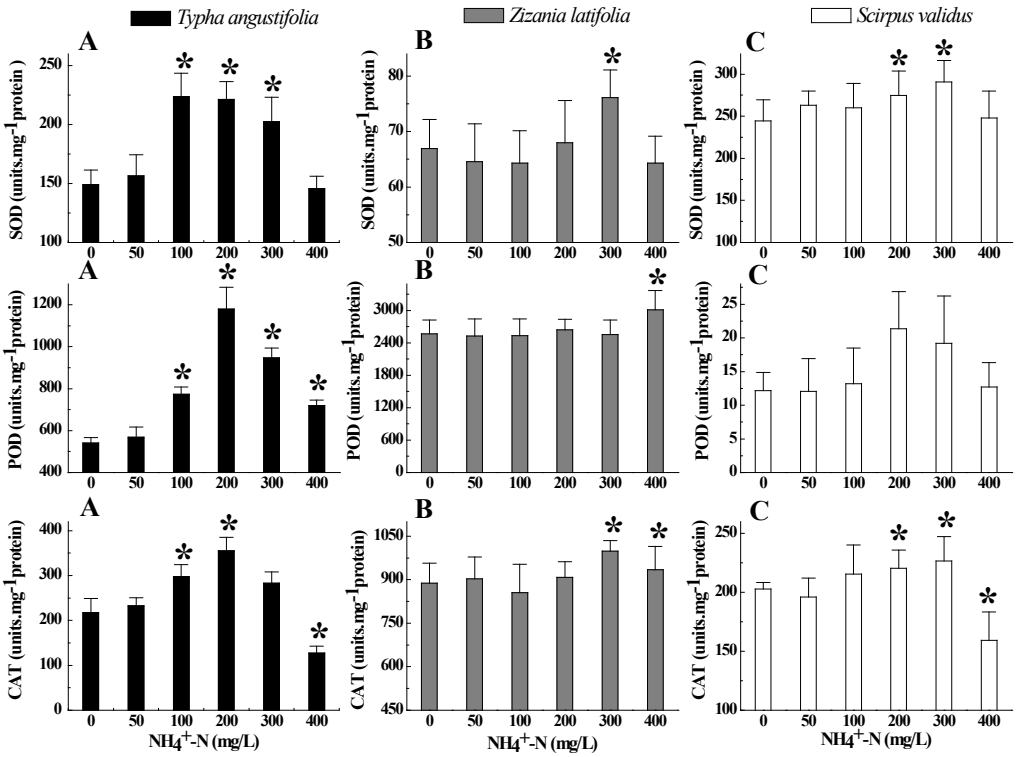

Fig. 2. Effects of different concentrations of ammonia on SOD, POD and CAT of (A) *T. angustifolia*, (B) *Z. latifolia* and (C) *S. validus*. Values are mean ± S.E (standard error). Asterisks (*) indicate that mean values are significantly different between ammonia treatments and controls ($n = 4$, $P < 0.05$).

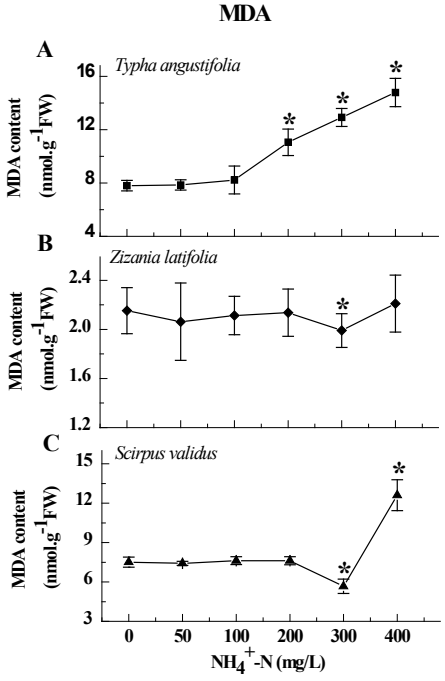

Fig. 3. Effects of different concentrations of ammonia on MDA in (A) *T. angustifolia*, (B) *Z. latifolia* and (C) *S. validus*. Values are mean ± S.E (standard error). Asterisks (*) indicate that mean values are significantly different between ammonia treatments and controls ($n = 4$, $P < 0.05$).

Chlorophyll a and b are the core of the photosynthetic system in plants, and the level of total chlorophyll can reflect the intensity of photosynthesis [24]. Several studies have reported that excess ammonia could inhibit photosynthesis efficiency by reducing the total chlorophyll content [8,12,13]. Results show that ammonia concentration above 100 mg·L^{-1} significantly reduced total chlorophyll content for *T. angustifolia*. Concentrations up to 400 mg·L^{-1} similarly reduced total chlorophyll content for *S. validus*. However, total chlorophyll content for *Z. latifolia* has not been greatly lowered from ammonia at the studied concentration range. These results showed that *Z. latifolia* has a highest ammonia tolerance, and *T. angustifolia* has a lowest ammonia tolerance in the three wetland species. And the variation of total chlorophyll content in three species demonstrated that ammonia stimulates chlorophyll content increase at moderate concentrations and reduced chlorophyll content at higher levels.

Reactive oxygen species (ROS) can be induced by a variety of stress, and high levels of ROS can damage proteins, DNA and lipids in plants. Thus, there are abundant antioxidants in plants used to scavenge excess ROS, and SOD, POD and CAT are the most effective antioxidants [25]. Recent studies have shown that ammonia stress can trigger the promotion of oxidative stress in plants by analyzing the activity changes of antioxidative enzymes

[9,10,12–14]. In our study, the SOD, CAT and POD activity increased significantly in *T. angustifolia, Z. latifolia* and *S. validus* under ammonia stress, so we speculated that ammonia could induce oxidative stress in the three wetland plants.

SOD forms the first line to remove ROS through dismutating superoxide (O_2^-) to H_2O_2. The initial increasing of SOD activity in three species appeared at different ammonia levels, with T. angustifolia at 100 mg·L^{-1}, *Z. latifolia* at 300 mg·L^{-1} and *S. validus* at 200 mg·L^{-1}. These findings indicated that excess ammonia could induce the SOD activity in these species, and different plants have different variation trends. However, SOD activity of the three species reduced to the level of controls under 400 mg·L^{-1} ammonia treatment, indicating that SOD activity may be suppressed by higher concentrations of ammonia. The similar results were found in the research of Wang et al. who studied the submerged plant exposed to high levels of ammonia [12].

POD and CAT can decompose H_2O_2 which is produced through dismutation of O_2^- by SOD into water and oxygen. In this study, the activity of POD in *T. angustifolia* and *Z. latifolia*, and that of CAT in all the three species increased significantly in response to excess ammonia. But, like SOD activity, the CAT activity was also suppressed at higher concentrations of ammonia (400 mg·L^{-1}) in *T. angustifolia* and *S. validus*, except *Z. latifolia*. Increased POD and CAT activity has also been reported in aquatic plants under ammonia stress [9, 12]. These results showed that, ammonia concentrations above 100 mg·L^{-1} could produce oxidative stress for *T. angustifolia*. Concentrations up to 200 mg·L^{-1} could cause oxidative stress for *S. validus*. And, concentrations in excess of 300 mg·L^{-1} could result in oxidative stress for *Z. latifolia*.

When ROS levels were above the capability of antioxidant system to scavenge, the lipid peroxides which can damage membranes and oxidize proteins in plants may be formed [25]. Malondialdehyde is a major product of polyunsaturated fatty acids peroxidation and has been used to measure the level of lipid peroxidation [26]. An accumulation of MDA was observed in *T. angustifolia* and *S. validus*, at ammonia concentrations up to 200 and 400 mg·L^{-1}, respectively. In contrast, MDA content for Z. latifolia was not significantly increased in the study. This indicated that *Z. latifolia* has a higher resistance capability under ammonia stress which provides a better protection from oxidative damage caused by excess ammonia. However, some reports found a reduction of MDA content in submerged plants under ammonia stress [9,12–13]. Thus the authors suggest that further experiments should be required to research the cause of difference in MDA content.

5. Conclusion

The results of this study indicate that excess ammonia can trigger oxidative stress and damage the photosynthetic system in wetland plants *T. angustifolia*, *Z. latifolia* and *S. validus*.

The different responses of chlorophyll and antioxidant system to ammonia stress by the three plants indicated that these plants had different ammonia tolerance. *Z. latifolia* has a high ammonia tolerance, and *T. angustifolia* has a low ammonia tolerance in the three wetland species.

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