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Tolerance and physiological responses of sweet flag (*Acorus calamus* L.) under nitrite stress during wastewater treatment

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ABSTRACT

Various newly discovered novel biological nitrogen removal (BNR) routes via nitrite offer great potential for improving nitrogen removal efficiency of constructed wetlands. In these novel routes, nitrite is required as the substrate or intermediary media. Hence, in the present study, the tolerance and physiological responses of sweet flag (*Acorus calamus* L.) to nitrite stress were investigated. Results showed that the physiology of sweet flag was not significantly affected when exposed to nitrite up to 20 mg/L. High nitrite levels (≥ 30 mg/L) led to changes in its cell membrane permeability, plant photosynthesis and activities of antioxidant enzymes. Particularly, when exposed to a nitrite level of 40 mg/L, sweet flag displayed trends of significant increase in the percentage of electrolyte leakage and contents of malondialdehyde and proline, whereas the chlorophyll content and plant height decreased and antioxidant defence systems were disrupted. Therefore, for CWs with sweet flag, 30 mg/L is the recommended threshold value for nitrite.

1. Introduction

Constructed wetlands (CWs), a green technology that requires less energy consumption, have been widely applied for purification of various wastewaters for several decades and consist of plants, substrates, microbes and wastewater. The sustainable operation of CWs depends on many aspects (e.g., plant growth, pollutant load, hydraulic retention time, running mode and structure design) (Kivaisi, 2001). Wetland plants have a positive effect on removal of contaminants and microbial abundance and community structure, which play a vital role in the healthy development of wetlands (Wang et al., 2016). Nitrogen (N) is effectively removed from wastewater in CWs by a variety of physical, chemical, and biological mechanisms (e.g., volatilization, ammonification, nitrification-denitrification, plant uptake and sediment adsorption) (Vymazal, 2013; Wu et al., 2015). Among these mechanisms, nitrification-denitrification is considered the primary process for N removal (Vymazal, 2007). Since the mid 1990s, CWs have been increasingly used as a low energy 'green' technique, but the nitrogen removal performance of the CWs is mostly poor. The average removal efficiency of NH_4^+-N and TN in 268 wetlands in Europe was 30% and 39.6%, respectively (Zhang et al., 2011). Vymazal (2007) found removal of TN in different CWs varied between 40% and 55% with the removed load ranging from 250 to 630 g N m^{-2} yr^{-1}, depending on the type of and inflow loading. Yang et al. (2008) found the removal efficiencies of TN and NH_4^+-N in pilot-scale mangrove wetland were 46% and 50% for municipal sewage treatment in Futian, Shenzhen, China.

Based on recent evidence, various novel biological nitrogen removal (BNR) routes observed in CWs provide significant potential for enhanced N removal and improved treatment performance: completely autotrophic nitrogen removal of nitrite (Canon), partial nitrification (PN) and anaerobic ammonium oxidation (Anammox) (Dong and Sun, 2007; Lee et al., 2009; Fu et al., 2017). Tao and Wang (2009) reported that the integration of PN and Anammox into CWs increases N removal efficiency without artificial aeration and also minimizes greenhouse gas emissions. Sun and Austin (2007), using a mass balance analysis, found that the CANON process in CWs converts N into gaseous form, which causes the loss of N mass and an increase in total nitrogen removal. The development of novel BNR routes in CWs are attracting great interest because of saved energy consumption without aeration (63%, 25% and 64% for CANON, PN and Anammox, respectively) (Ren et al., 2016; Third et al., 2001; Wei et al., 2015), reduced demand for an external organic carbon source, and minimized greenhouse gas release (Lee

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et al., 2009). However, among all these BNR routes, nitrite is required as the substrate or intermediary media, which is well-known to destroy plant physiological functions and inhibit photosynthesis, stimulate the generation of reactive oxygen species (ROS), and increase the proton permeability of membranes (Vadivelu et al., 2006; Singh et al., 2007). Thus, the tolerance of wetland plants to nitrite stress must be determined to develop novel BNR routes in CWs. The physiological responses of plants to pollutants and growth conditions have been studied and extensively reviewed for many years. These studies have been successful in determining the effects on plants and the resistance mechanisms to pollutants and conditions such as high nitrogen (NH_4^+ and NO_3^- forms) and phosphorus (PO_4^{3-}) concentrations (Vojtíšková et al., 2004; Su et al., 2012), low oxide content (Schlüter and Crawford, 2001), low light availability (Zhang et al., 2010), excess heavy metals (Cu, Zn, Pb, Cd, Cr) (Zhou et al., 2007; Liu et al., 2016; Reale et al., 2016), fluctuations in water levels (Wei et al., 2014), the combined effect of salinity and high pH (Calvo-Polanco et al., 2014), fluoroquinolone antibiotics (Riaz et al., 2017) and persistent organic pollutants (naphthalene) (Lai et al., 2016). To date, the tolerance and physiological responses of plants under nitrite stress in CWs have not been fully evaluated. Thus, the physiological mechanisms and stress tolerances of wetland plants to excess nitrite concentrations should receive increased attention.

Sweet flag (*Acorus calamus* L.) is widely used in the purification of wastewater as an emergent macrophyte colonizing littoral zones of eutrophic habitats in CWs (Brändle et al., 1996). Moreover, sweet flag also has high tolerance for ammonia and anoxia (Crawford and Braendle, 1996; Weber and Brändle, 1996). These characteristics are advantages for the invasion of sweet flag and establishment in competition with other wetland plant species.

Therefore, the objective of the current study was to determine the nitrite tolerance and associated physiological mechanisms of sweet flag and to propose the threshold value of nitrite for the development of novel biological nitrogen removal routes in wetlands.

2. Materials and methods

2.1. Plant and growth conditions

Sweet flag was transplanted from Nansi Lake in Shandong Province in November 2016. The plants were transplanted carefully to avoid damaging to the plant roots. Nansi Lake is an important storage lake in the east route of the South-to-North Water Diversion Project in China. Historically, the lake had been serious polluted, however, it was well restored thorough extensive promotion of constructed wetlands, making it a typical site for CW study in northern China. The weather during the study period was find and the temperature was around 5–10 °C. The sampled plants were put outdoors for about one week. In the present experiment, the plants selected were one-year, healthy and similar in size and were cultivated in plastic buckets (20 cm in depth and 25 cm in diameter) with sand culture (15 plants per pot). In December, plants were transferred to indoor breeding in Jinan in north China (36°40′36″ N, 117°03′42″ E), under temperatures of 17/15 °C (day/night), a light/dark cycle of 12/12 h and 40% humidity. Approximately 600 μmol m^{-2} s^{-1} PAR (photosynthetically active radiation) was natural daylight, which was provided by photosynthetic photon flux density grow lights from above the plants. After planting, the plants were watered with tap water and 10% Hoagland solution (Xu et al., 2010) for two weeks before the experiment. The 10% Hoagland solution had the following composition: 0.6 mM KNO_3, 0.4 mM $(CaNO_3)_2{\cdot}4H_2O$, 0.1 mM $NH_4H_2PO_4$, 0.2 mM $MgSO_4{\cdot}7H_2O$, 0.9 μM $MnCl_2{\cdot}4H_2O$, 0.46 μM H_3BO_2, 0.08 μM $ZnSO_4{\cdot}7H_2O$, 0.03 μM $CuSO_4{\cdot}5H_2O$, 0.01 μM $H_2MoO_2{\cdot}H_2O$, and 0.09 μM Fe–EDTA. Each of the treatment buckets was filled with 5 cm of gravel (3–4 cm in diameter) as the supporting layer and 10 cm of sand (1–2 mm in diameter, collected from the Yellow River) for promoting the dispersion of sewage and growth of plants.

Plants were exposed to increasing NO_2-N concentrations of 0 (control), 5, 10, 20, 30, and 40 mg/L, which were prepared by mixing $NaNO_2$ into 10% Hoagland solution (two replicates). To minimize variability, the wastewater with nitrite was replaced every 2 days. The physiological changes in the plants were observed every 5 days, and all plants were treated for 20 days. Plant leaves were harvested randomly from the different groups and were rinsed with distilled water and immediately weighed. Then, leaf samples were immediately stored in liquid nitrogen at -80°C for further analysis.

2.2. Determination of photosynthetic pigments

The leaf samples were cut into 1–2 cm square pieces and extracted for 24 h in darkness with 25 mL of 80% acetone. Then, the total chlorophyll content of leaves was determined using an ultraviolet spectrophotometer at 652 nm, as described by Bruuinsma (1963). All analyses were conducted in triplicate. The chlorophyll content was expressed based on fresh weight (FW) (mg/g FW).

2.3. Determination of the percentage of electrolyte leakage (PEL)

The leaf samples were washed and dried on filter paper, immediately weighed and cut into segments (ca. 1 cm). To determine the primary electrolyte leakage (K1), one hundred micrograms of fresh leaf sample was added to 10 mL of diluted water for 12 h. Then, the leaves in solution were heated in boiling water for 30 min. The leaves were removed, and the solutions were cooled to room temperature to measure the final electrolyte leakage (K2). The K1 and K2 were measured using a conductivity meter (DDBJ-350; Leici, China). The PEL was calculated using the following equation: $P = K1/K2 \times 100\%$ (Yin et al., 2016).

2.4. Determination of malondialdehyde (MDA) and proline contents

To estimate the degree of lipid peroxidation, the accumulation of MDA was determined according to the instructions of a determination kit (A003-1; Nanjing Jiancheng Bioengineering Institute; NJBI). MDA was measured using the thiobarbituric acid (TBA) method, with MDA reacting with TBA to produce a red substance. The absorbance of the supernatant was measured at the wavelength 532 nm. The MDA content was expressed as nmol per g fresh weight (FW) of leaves. Proline content in leaves was measured as described by a determination kit (A107; Nanjing Jiancheng Bioengineering Institute; NJBI). Proline reacts with acid ninhydrin generating stable red products. The top layer of fluid was obtained and the absorbance measured at 520 nm. The proline content was expressed as μmol per g FW of leaves.

2.5. Assays of the activities of antioxidant enzymes

The enzymatic defence system includes antioxidant enzymes such as total superoxide dismutase (T-SOD), catalase (CAT), and peroxidase (POD), which were assayed by determination kit Nos. A001-1, A007-1, and A084-3, respectively (NJBI). One unit of T-SOD activity was defined as the amount of plant extract that inhibited the rate of xanthine reduction by 50%. One unit of CAT activity was defined as the amount of enzyme that catalysed the decomposition of 1.0 μmol of H_2O_2 per min. One POD unit was defined as the amount of enzyme required to decompose 1 μg of substrate per min. T-SOD, POD, and CAT activities of plant leaves were expressed as unit activity per g FW of leaves.

2.6. Statistical analyses

All statistical analyses were performed using the SPSS statistical software package 11.0 (SPSS Inc., Chicago, USA). Two-sample t-tests were used to evaluate the significance of differences among means. The