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Ammonium-induced oxidative stress on plant growth and antioxidative response of duckweed (*Lemna minor* L.)

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ABSTRACT

This study investigates ammonium (NH_4^+) uptake kinetics, growth characteristics, and antioxidative response of *Lemna minor* L. to NH_4^+ exposure. *L. minor* was exposed to different NH_4^+ levels (0.5, 1, 2, 3, and 4 mM) for various contact durations (1, 3, 5, 9, and 14 d). *L. minor* grew well in 0.5–3.0 mM NH_4^+, with a relative growth rate of 0.046–0.048 d^{-1}. The maximum uptake velocity was achieved at 0.066 mg g^{-1} FW h^{-1}, and the growth velocity could be fitted well with the Michaelis–Menten function (R^2 = 0.90507). Plant growth was suppressed, and the chlorophyll and carotenoid contents decreased after "14"-day of exposure on 4 mM NH_4^+. 4 mM NH_4^+ induced oxidative stress and heated the antioxidative response in *L. minor* after "5"-day of exposure. Superoxide dismutase activity increased in contrast to the peroxidase activity decreasing compared with control. The increased malondialdehyde indicated membrane lipid peroxidation because of incomplete antioxidative reactions.

1. Introduction

The sustainable growth of industry and agriculture has attracted a great deal interest among environmental protection organizers due to the development of highly cost-effective nutrient management technologies (Xu and Shen, 2011). Among these technologies, the conversion of nutrients into valuable plant biomass has drawn increasing attention for its two advantages: effective pollutant removal and nutrient recycling from postharvest biomass (Mohedano et al., 2012). Aquatic plants are important because they produce oxygen, and they are a source of food and provide protection for other organisms, keeping aquatic ecosystems healthy by accumulation or decomposition of toxins (Wang et al., 2010). Ammonium (NH_4^+) and nitrate (NO_3^-) are the most important dissolved inorganic nitrogen in aquatic ecosystems. Moreover, NH_4^+ assimilation requires less energy than NO_3^-, thus, several plants prefer NH_4^+ as their nitrogen source (Miller and Cramer, 2005).

Several species of aquatic macrophytes choose NH_4^+ as their inorganic nitrogen source (Jampeetong and Brix, 2009). However, these species differ in their NH_4^+ toxicity tolerance; some species are inclined to avoid from uptake of high NH_4^+ by physiological changes (e.g. shallow rooting) (Tylova et al., 2008). NH_4^+-induced toxicity symptoms in plants have been observed for at least a century (Britto and Kronzucker, 2002). The impacts of NH_4^+ supply on metabolism have also been pointed out in submersed and floating aquatic plants (Miller and Cramer, 2005; Njambuya et al., 2011).

Oxidative stress can be induced by oxygen deprivation including the direct photoreduction of O_2 to O_2^- by reduced electron transport associated with the photo-respiratory cycle (Wang et al., 2008). Reactive oxygen species (ROS) can be induced by various stresses (Mittler et al., 2004); excessive ROS can result in oxidative damage to proteins, DNA, and lipids. The production of ROS is one of the main causes for productivity decreases, injury, and death that accompany these stresses in plants. The mechanisms existing in plant cells can also be stimulated to regulate the overproduction of ROS, counteracting the created oxidative stress. These mechanisms include antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbic peroxidase, and dehydroascorbate reductase (Wang et al., 2010). Levels of H_2O_2 and O_2^- are better controlled by antioxidant enzymes or antioxidants.

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However, excess H_2O_2 and O_2^- are transformed to hydroxyl radicals via the Haber–Weiss reaction, thereby leading to lipid peroxidation through the degradation of polyunsaturated fatty acids (Apel and Hirt, 2004). Malondialdehyde (MDA) is a secondary end-product of polyunsaturated fatty acid oxidation and has been used to indicate the degree of membrane lipid peroxidation (Wang et al., 2008).

Duckweed is a small, floating aquatic plant within the family Lemnaceae. It has no true roots, but has a submerged, thin, root-like, white culm, which probably has similar functions as actual roots. The geographic ranges of duckweed span the entire globe; 40 species belonging to four genera (*Lemna*, *Spirodela*, *Wolffia*, and *Wolffiella*) have been identified so far (Bergmann et al., 2000). *Lemna minor* L. has the potential for wastewater treatment because it has a high growth rate in nutrient-rich and stagnant waters (Alvarado et al., 2008) and as the produced biomass can easily be harvested. There is some information available regarding the growth and ecology of this species that suggests preference for growth in acidic waters (Körner et al., 2001), and preference for NO_3^- over NH_4^+ as the inorganic N-source (Britto and Kronzucker, 2002), although duckweed has a preferential uptake of NH_4^+ (Mohedano et al., 2012). The main reason was that NH_4^+ might be directly related to the glutamine synthetase/glutamate synthase enzyme system to synthetize protein, while NO_3^- should be firstly transferred to NH_4^+ by nitrate/nitrite reductase enzyme system (Takahashi and Mercier, 2011). Thus, NH_4^+ assimilation requires less energy than NO_3^-, but NH_4^+ assimilation can induced the toxicity symptoms in some plants.

Scholars hold different views on duckweed tolerance to NH_4^+ toxicity. Xu and Shen (2011) reported that duckweed has a preferential NH_4^+ uptake according to their full-scale experiments on swine wastewaters treated with *Spirodela oligorrhiza*. Suppadit (2011) showed that a 12.0 g biomass of *Wolffia arrhiza* per liter of farm effluent and a 30-day treatment period provide the best conditions for the growth and quality of the treated farm effluent in terms of NH_4^+. Similar results showed that Lemnaceae can grow normally with high NH_4^+ uptake rates in duckweed-treated sewage (Cheng et al., 2002). Körner et al. (2001) found that the relative growth rates (RGRs) of *Lemna gibba* in domestic wastewater decreased at 80 mg L^{-1} NH_4^+. Cedergreen and Madsen (2002) reported that NH_4^+ uptake rates by the floating macrophyte *L. minor* was reduced when NH_4^+ was above 50 mg L^{-1}.

High amounts of NH_4^+ in wastewater may induce osmotic stress in plants, similar to the way high salt concentrations lead to hyperosmotic stress, and immediately affect growth, reduce cell expansion in young leaves, and cause antioxidative responses. Although several studies related to antioxidative responses of duckweed under high concentrations of heavy metals (Razinger et al., 2008), organic compound (Radić et al., 2011), surface active agent (Forni et al., 2012), and pesticide (Mitsou et al., 2006) have been published, a better understanding of the responses of duckweed to high NH_4^+ concentrations are needed to optimize its use in ecological system restorations or wastewater treatment processes based on *L. minor*. This study is focused on the growth and physiological responses of *L. minor* to high NH_4^+ concentrations to understand its performance in a highly loaded and NH_4^+-rich water body, reflecting the tolerance capacity to resist elevated NH_4^+ levels.

2. Materials and methods

2.1. Plant culture and treatments

L. minor was collected from a small, eutrophic pond in Chongqing, PR China. The plants were brought to the laboratory, rinsed with 2% (M/V) sodium hypochlorite, and then placed in several tanks (50 cm long, 35 cm wide, and 10 cm high) containing an artificial growth medium. These tanks were kept in a greenhouse at day/night temperatures of 23 ± 2 °C (Ge et al., 2012). Light was provided by metal halide bulbs (Osram, 250 W) at a photon flux density of 3000 ± 500 lx in a 16 h light/8 h dark cycle (Kim et al., 2012; Njambuya et al., 2011). The plants were cultured for two weeks in an improved Hoagland solution (Tkalec et al., 1998) for acclimatization and amplification prior to the experiments. The experimental growth medium was changed for every two days. The improved Hoagland solution had the following composition: 252.5 mg L^{-1} KNO_3; 542.8 mg L^{-1} $Ca(NO_3)_2{\cdot}4H_2O$; 246 mg L^{-1} $MgSO_4{\cdot}7H_2O$; 68 mg L^{-1} KH_2PO_4; 1.43 mg L^{-1} H_3BO_3; 0.91 mg L^{-1} $MnCl_2{\cdot}4H_2O$; 0.11 mg L^{-1} $ZnSO_4{\cdot}7H_2O$; 0.05 mg L^{-1} $Na_2MoO_4{\cdot}2H_2O$; 0.05 mg L^{-1} $CuSO_4{\cdot}5H_2O$; 9.68 mg L^{-1} $FeCl_3{\cdot}6H_2O$; and 30 mg L^{-1} $EDTANa_2$. Healthy plants were cultivated in a growth chamber (Light incubator PGX-430B, China) at 23 ± 0.5 °C and a photon flux density of 3000 ± 100 lx in a 16 h light/8 h dark cycle after the amplification culture. The growth medium was a nitrogen-free, 1/10 improved Hoagland solution added with various concentrations of NH_4^+. The experimental treatments consisted of 11 levels of NH_4^+ concentrations (0.5, 1, 3, 5, 7, 10, 14, 28, 42, 56, and 70 mg·L^{-1}) for measuring uptake kinetics, and 5 levels of NH_4^+ concentrations prepared from NH_4Cl (0.5, 1, 2, 3, and 4 mM) for measuring plant growth and physiological responses. The pH of the growth medium was adjusted to 7.0 ± 0.5 using a pH meter (PB-100, Germany) (Ge et al., 2012).

The growth medium was changed every two days during the plant growth and physiological response experiments. The plants were harvested and cleaned after treatment for certain number of days. Their fresh weights (FWs) were measured after blotting with tissue paper. The RGR (g g^{-1} d^{-1}) in each treatment was calculated by the formula $RGR = \ln W2 - \ln W1)/t$, where $W1$ and $W2$ are the initial and final FW (g), and t is the incubating time (d) (Jampeetong and Brix, 2009).

2.2. Ammonium uptake kinetics

NH_4^+ uptake rates were measured under light and temperature conditions similar to growth conditions. About 1.0–2.0 g FW plant material was chosen and pre-incubated in 1-L beakers with 1000 mL nitrogen-free growth medium for 48 h. Uptake kinetics were determined by incubating in a 1-L beaker with 200 mL growth medium of different NH_4^+ levels for 8 h. The NH_4^+ uptake rates (ν) were calculated by the formula $\nu = (C_0 - C_t)V/t$, where C_0 and C_t are the initial and final NH_4^+ concentration (mg L^{-1}), V is the growth medium volume, and t is the incubating time (h). The Michaelis–Menten (M–M) function (Barber, 1979) was applied using experimental data to evaluate the uptake kinetics of NH_4^+. The M–M equation is expressed as follows:

$$\nu = \nu_{muc}\frac{C_0}{K - C_0}$$

where ν and ν_{muc} (mg g FW h^{-1}) are the NH_4^+ uptake rates and maximum uptake capacity, respectively, and K (h^{-1}) is the Michaelis–Menten half-saturation constant.

2.3. Enzyme extraction, protein and photosynthetic pigment contents

About 500 mg (FW) *L. minor* was homogenized in 5 mL cold potassium phosphate buffer (0.1 M, pH 7.8) to obtain the enzyme extract. The homogenate was centrifuged at 15,000 × *g* (4 °C) for 15 min. The supernatant was used as enzyme extract. All steps for enzyme extract preparation were carried at 4 °C. Protein content was quantified according to the Bradford Method (1976).