Chemosphere 86 (2012) 538–545

Contents lists available at SciVerse ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Physiological responses of *Egeria densa* to high ammonium concentration and nitrogen deficiency

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ARTICLE INFO

Article history:
Received 16 March 2011
Received in revised form 14 October 2011
Accepted 20 October 2011
Available online 17 November 2011

Keywords:
Ammonium
Nitrogen deficiency
Aquatic plants
Egeria densa
Physiological stress

ABSTRACT

High ammonia (i.e. the total of NH_3 and NH_4^+) concentration or nitrogen deficiency in water can exert stress on growth and health of many aquatic plants. To investigate the physiological impacts of high ammonia-N (NH_4Cl) concentration and nitrogen deficiency on plant physiology, apical shoots of submerged macrophyte *Egeria densa* were first treated with five levels of nitrogen: 0, 1, 10, 30, 60 mg L^{-1} ammonia-N (NH_4Cl) for 5 d. After having explored the stress range of ammonia-N, its effect on *E. densa* was further examined at three levels of ammonium (0, 1, 30 mg L^{-1} ammonia-N) and at six exposure times (0, 1, 2, 3, 5 and 7 d). In testing the concentration-dependent stress, the increase of ammonia-N reduced the amounts of total chlorophyll (chl *a* and *b*), soluble proteins and soluble carbohydrates, but increased the activity levels of malondialdehyde (MDA), superoxide dismutase (SOD), catalase and peroxidase in *E. densa*. In the N-free medium, total chlorophyll, soluble proteins, soluble carbohydrates and the activities of SOD and peroxidase in *E. densa* decreased significantly compared with the control (1 mg L^{-1} ammonia-N). When comparing the ammonia-N impacts over time, the plants showed a declining trend in total chlorophyll, soluble proteins and soluble carbohydrates, but an rising trend in MDA, SOD, peroxidase and catalase in 30 mg L^{-1} ammonia-N over 7 d. Compared with the control, the N-free medium significantly decreased the amounts of total chlorophyll, soluble proteins, soluble carbohydrates, SOD and peroxidase in *E. densa* over time. Our study indicates that high ammonium (ammonia-N $\geqslant$ 10 mg L^{-1}) affects the growth of *E. densa* through inducing oxidative stress and inhibiting photosynthesis, and nitrogen deficiency can also induce an abiotic stress condition for the *E. densa* growth by reducing photosynthetic pigments, soluble proteins, soluble carbohydrates, and the activity of antioxidant enzymes.

1. Introduction

In a changing environment, plants are inevitably exposed to various stress conditions. A stress situation does not only impose a prominent effect on plant growth, development and distribution (Litav and Agami, 1976; Ni, 2001; Britto and Kronzucker, 2002; Camargo and Alonso, 2006; Cao et al., 2007a; Nimptsch and Pflugmacher, 2007), but also induces reactive oxygen species (ROS) in cells and tissues to cause damage (Foyer et al., 1994; Huang et al., 2004). In plant cells, superabundant ROS including superoxide radical ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) can damage tissues and seriously disrupt metabolism through oxidation to membrane lipids, proteins, pigments and nucleic acids (Misra and Gupta, 2006). To eliminate or reduce ROS, plants have evolved various protective mechanisms. Hereinto, anti-oxidant enzymes such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) compose a key protective anti-oxidant defense system in plant. The capacity of the anti-oxidant defense system often increases under a stress condition (Rout and Shaw, 2001; Misra and Gupta, 2006). In plants, therefore, fluctuations of many metabolites such as chlorophyll (Ferrat et al., 2003), soluble proteins (Amini and Ehsanpour, 2005), soluble carbohydrates (Costa and Spitz, 1997; Sativir et al., 2000), malondialdehyde (Bailly et al., 1996; Chen et al., 2003), and anti-oxidant enzymes are important indexes to measure the response of physiological oxidative stress.

Ammonia (i.e. total NH_3 and NH_4^+) is an important nitrogen source for plant growth and development. However, excessive ammonia often presents a stress condition for plant growth (Britto and Kronzucker, 2002). Total ammonia in aqueous solution consists of ammonium ion (NH_4^+) and un-ionized ammonia (NH_3), with relative concentrations being regulated by pH and temperature.

Abbreviations: MDA, malondialdehyde; SOD, superoxide dismutase; ROS, reactive oxygen species; $O_2^{\cdot}$, superoxide radical; H_2O_2, hydrogen peroxide; $OH^{\cdot}$, hydroxyl radical.

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0045-6535/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.chemosphere.2011.10.036

Both forms are reported to become toxic at higher concentrations (Litav and Agami, 1976; Körner et al., 2001). In an aquatic environment, ammonia derived from agricultural run-off, atmospheric deposition, industrial discharge and urban sewage can reach 10–200 mg L^{-1} (Körner et al., 2001). High ammonia concentration often triggers eco-physiological stress on aquatic plants, resulting in a decline or loss of macrophytes (Litav and Agami, 1976; Ni, 2001; Camargo and Alonso, 2006; Cao et al., 2007a; Nimptsch and Pflugmacher, 2007). High ammonia can negatively impact survival, growth and reproductive capacity in plant (Best, 1980; Ni, 2001; Cao et al., 2007a; Li et al., 2007), and reduce the contents of chlorophylls (Wang et al., 2008; Wang et al., 2010), soluble proteins and soluble carbohydrates in plant tissues (Cao et al., 2004; Cao et al., 2007b; Yan et al., 2007). High ammonia often increases malondialdehyde (MDA) content (Cao et al., 2009; Jiao et al., 2009) in plant. Moreover, ROS induced by high ammonia stress can lead to the changes of antioxidant enzyme activities including SOD, CAT and POD for plant to cope with stress (Nimptsch and Pflugmacher, 2007; Wang et al., 2008; Cao et al., 2009; Wang et al., 2010). Previous studies have showed that the toxicity of 1.5–28 mg L^{-1} ammonia-N to aquatic plants can be detected from 4 to 8 d of exposure, but toxic responses are species-specific (Nimptsch and Pflugmacher, 2007; Wang et al., 2008). However, the stress effect of high ammonia on aquatic plants that have potential use for environmental remediation in populated water is little known (Nimptsch and Pflugmacher, 2007).

N deficiency is also a common stress for growth of many plants (Huang et al., 2004; Polesskaya et al., 2004). In terrestrial plants, N deficiency can suppress plant growth by reducing the photosynthetic capacity of leaves (Verhoeven et al., 1997; Lu and Zhang, 2000; Milroy and Bange, 2003; Huang et al., 2004), degrade photosynthetic pigments and proteins (Huang et al., 2004; Polesskaya et al., 2004), and reduce enzyme synthesis in plants (Verhoeven et al., 1997; Huang et al., 2004; Polesskaya et al., 2004). However, little is known on the role of N deficiency in regulating chlorophyll content, metabolites and antioxidant enzymes in aquatic plants.

Egeria densa (Planch), commonly known as Brazilian waterweed, is a widely used plant in ornamental fish industry in the world via aquarium trades (Haramoto and Ikusima, 1988). It lives in an environment with moderately high light intensity, and conducts photosynthesis in stems and leaves. Because of its ease in propagation through double node, root crown or apical shoot (Getsinger and Dillon, 1984; Haramoto and Ikusima, 1988) and high capacity to absorb nutrients such as ammonium and phosphorus from water column (Feijoó et al., 2002), *E. densa* has been used as a model plant to assess water quality, heavy metal accumulation and toxicity, and metabolism of pesticides in plants (Malec et al., 2009). *E. densa* is able to live in 6 mg L^{-1} ammonia-N, which sheds a hope as a candidate to remove ammonia from polluted water (Feijoó et al., 2002). However, the ammonia tolerance of this plant has not been further examined.

In this study, we tested the hypothesis that either high ammonium or nitrogen deficiency stresses this model species of aquatic plants through regulating leaf chlorophyll content, metabolite levels, and plant growth. We first treated the *E. densa* apical shoots with a wider range of NH_4Cl concentrations and described the plant response at the end of the experiment. Then, the plants were treated with a narrow range of NH_4Cl concentrations, but with more frequent observations over time. In the whole experiment, NO_3^- was excluded in the culture medium. The objectives of this study were (1) to investigate the effects of excessive ammonia in water on the content of total chlorophyll (i.e. chl *a* and *b*), soluble proteins, soluble carbohydrates, MDA and the activity of antioxidant enzymes (SOD, catalase and peroxidase) in *E. densa*; and (2) to evaluate the impact of nitrogen deficiency on the physiological stress response of *E. densa*.

2. Materials and methods

2.1. Plant material and experimental design

The *E. densa* were obtained from an aquarium shop in Chongqing, China. Plants were cultured for over 2 weeks in two aquariums (50 × 40 × 40 cm) nourished with Tetra Plant Florapride (Tetra Werke Company, Germany). Prior to exposure, 8 cm of apical shoots were acclimatized to 1 mg L^{-1} (N) NH_4Cl for 5 d in 1-L glass beakers containing the primary culture medium. All beakers were placed in controlled incubators with a photoperiod of 12 h light and 12 h dark, temperature 25 ± 0.2 °C and 6500 lx light intensity.

The other ingredients of the culture medium included 1% Hoagland's trace elements (Hoagland and Arnon, 1950) and the macro-nutrition elements adapted from the recipe of Smart and Barko (1985). The N-free macro-nutrition medium was composed of 0.008 mM KH_2PO_4, 0.4 mM $MgSO_4{\cdot}7H_2O$, 0.5 mM $CaCl_2{\cdot}2H_2O$ and 0.5 mM $CaCO_3$.

The minimum requirement of ammonia for the normal growth of *E. densa* was above 1 mg L^{-1} ammonia (Feijoó et al., 2002). Therefore, the range of 1–60 mg L^{-1} (N) NH_4Cl was chosen in this study to investigate the stress of high ammonia on *E. densa* and 1 mg L^{-1} ammonia-N was set as a control representing the nutrient level at which normal growth was sustained. Meanwhile, the treatment of nitrogen free (N-free) was set to compare the stress of nitrogen deficiency on *E. densa* with that of normal ammonia supply.

The whole study consisted of a concentration-dependent experiment and a duration-dependent experiment. The former included treatments of 1(control), 10, 30, 60 mg L^{-1} (N) NH_4Cl and N-free, and the experiment was terminated by d 5 after treatment. The latter narrowed the range of the ammonia concentrations to 1(control), 30 mg L^{-1} (N) NH_4Cl and N-free, but the plants were sequentially sampled on 1, 2, 3, 5, and 7 d after treatment.

2.2. Plant exposure and sampling

The ammonia stock solution (1000 mg L^{-1} N) was prepared by dissolving 3.9 g NH_4Cl per liter in de-ionized water. After acclimation, 10 *E. densa* apical shoots of an equal size were planted in one beaker in triplicate.

In the concentration-dependent experiment, after the plant biomass was weighed and recorded, the apical shoots were treated with 1, 10, 30, 60 mg L^{-1} (N) NH_4Cl and N-free in triplicate. Throughout the acclimation and exposure periods, the nutrient solution was renewed every 2 d and the pH of the solution was adjusted to 7.0 ± 0.1 with 0.5 M H_2SO_4 or 1 M NaOH twice a day. At the end of 5-d exposure period, plants in each beaker were harvested, rinsed with distilled water and blotted dry, and then the plant biomass was weighed to evaluate plant growth. Then, two apical shoots from each replicate were randomly collected and pooled into one sample (2.0–2.3 g) for biochemical measurements. All of the samples were stored at −20 °C before biochemical analyzes.

In the time-dependent experiment, the plants were exposed to 1, 30 mg L^{-1} (N) NH_4Cl in triplicate and an N-free medium, and were sampled on 1, 2, 3, 5, and 7 d. On each sampling day, two apical shoots from each replicate were randomly collected, rinsed with distilled water, blotted dry, and pooled into one sample (2.0–2.3 g) for biochemical measurements. The renewal of nutrient solution and the management of pH were the same as above.

According to the formula developed by Caicedo et al. (2000), the proportion of NH_3-N was <0.6% of the total nitrogen in the culture media. To confirm the actual ammonia-N concentrations used in our experiment, for each test solution, water samples were taken at the beginning and the end of the exposure period. The concentration of ammonia was measured using the water quality measuring