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Modulation of cadmium-induced phytotoxicity in *Cabomba caroliniana* by urea involves photosynthetic metabolism and antioxidant status

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ABSTRACT

Urea is a widespread organic pollutant, which can be a nitrogen source, playing different roles in the growth of submerged macrophytes depending on concentrations, while high cadmium (Cd) concentrations are often toxic to macrophytes. In order to evaluate the combined effect of urea and Cd on a submerged macrophyte, *Cabomba caroliniana*, the morphological and physiological responses of *C. caroliniana* in the presence of urea and Cd were studied. The results showed that high concentrations of urea (400 mg L^{-1}) and Cd (500 μmol L^{-1}) had negative effects on *C. caroliniana.* There were strong visible symptoms of toxicity after 4 days of exposure under Cd-alone, 400 mg L^{-1} urea, and Cd + 400 mg L^{-1} urea treatments. In addition, 400 mg L^{-1} urea and Cd had adverse effects on *C. caroliniana*'s pigment system. Significant losses in chlorophyll fluorescence and photosynthetic rates, as well as Rubisco activity were also observed under Cd-alone, 400 mg L^{-1} urea, and Cd + 400 mg L^{-1} urea treatments. 400 mg L^{-1} urea markedly enhanced Cd toxicity in *C. caroliniana*, reflected by a sharp decrease in photosynthetic activity and more visible toxicity symptoms. The results of thiobarbituric acid reactive substances (TBARS) pointed to extreme oxidative stress in *C. caroliniana* induced under Cd or 400 mg L^{-1} urea exposure. Exogenous ascorbate (AsA) protected *C. caroliniana* from adverse damage in 400 mg L^{-1} urea, which further corroborated the oxidative stress claim under 400 mg L^{-1} urea. However, results also demonstrated that lower urea concentration (10 mg L^{-1}) alleviated Cd-induced phytotoxicity by stimulating chlorophyll synthesis and photosynthetic activity, as well as activating the activity of catalase (CAT) and glutathione-S-transferase (GST), which may explain the alleviating effect of urea on *C. caroliniana* under Cd stress.

1. Introduction

In natural ecosystems, plants are exposed to complex actions of organic and inorganic pollutants, which can induce both synergistic and antagonistic effects on the plants (Chukina and Borisova, 2010; Maleva et al., 2012). Cadmium (Cd), classified as a human carcinogen (Waisberg et al., 2003), is discharged and dispersed into aquatic ecosystems mainly through industrial processes or via the application of phosphate fertilizers (Mishra et al., 2006). High Cd solubility in water facilitates its wide distribution in aquatic systems, and its relatively high mobility in comparison to other metals in plant systems makes it a metal of major concern with respect to both plant exposure and human food chain contamination (Clemens, 2006). Plants, can readily take up Cd leading to phytotoxicity, with regard to genetic, biochemical and physiological changes that occur in plants (Mishra et al., 2006; Ding et al., 2007). Toxicity of Cd may result from its binding to sulfydryl groups of proteins leading to inhibition of activity or disruption of structures, disturbance of cellular redox control (Schutzendubel et al., 2002), and/or stimulation of production of reactive oxygen species (ROS) (Fodor, 2002; Sandalio et al., 2009; Andresen and Küpper, 2013). In response to Cd stress, plants have evolved different detoxification mechanisms. For instance, marked synthesis of phytochelatin was observed in *Pistia stratiotes*, whereas in *Eichhornia crassipes*, the antioxidant defense system was activated under Cd exposure (Sanita di Toppi et al., 2007). Among the various antioxidants, ascorbate (AsA), reduced glutathione (GSH), and the main antioxidant enzymes, i.e., superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD), are particularly important (Schutzendubel and Polle, 2002).

Elevated concentrations of nitrogen compounds in water bodies in urbanized territories have deleterious effects on plant populations. Urea is one of the major organic pollutants, characterized by its unique physiochemical properties, including non-polarity, high solubility, and

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rapid absorption. Due to the above properties, urea is considered as a more effective fertilizer than nitrates and ammonium. Global urea production is approximately 70 million metric tons per year, and is predicted to exceed 200 million metric tons per year by 2020 (Glibert et al., 2006). Increasing urea usage contributes to high available nitrogen concentrations in natural waters. Significant amounts of urea (generally 0.25–1.0 mg urea-N L^{-1}), originating from both natural and anthropogenic sources, end up in aquatic ecosystems (Glibert et al., 2006; Finlay et al., 2010).

Several studies have examined the effects of urea-import on the physiology of aquatic plants. Our previous study found that certain concentrations of urea could stimulate the synthesis of photosynthetic pigments and increase photosynthesis in *Cabomba caroliniana* and *Elodea nuttallii*. However, high concentrations of urea impaired growth in the two aquatic species (Huang et al., 2016), which is consistent with the results for *Elodea densa* (Maleva et al., 2013, 2015). Excessive application of urea (more than 100 mg L^{-1}) could decrease photosynthetic activity and the toxic mechanisms of urea on *E. densa*, based on the accumulation of ROS in leaves, which disturbs the balance and lead to oxidative stress (Maleva et al., 2013, 2015).

Investigations over the last several years present ample data regarding the separate influences of Cd or urea on the physiology of aquatic plants (Xu et al., 2012; Maleva et al., 2013, 2015; Chen et al., 2016; Huang et al., 2016). Due to excessive application, urea may be washed off from the agricultural fields, or move with water through leaching and erosion, enter and mix with Cd-loaded water bodies. In general, urea concentrations in water bodies would not exceed 1.0 mg L^{-1}. Nevertheless, under some conditions, such as fertilizer application prior to rainfall or irrigation, urea concentrations can greatly exceed 1 mg L^{-1} in aquatic ecosystems (Glibert et al., 2006; Huang et al., 2016). Thus, contamination of surface waters by the two factors represents a complex form of pollution, characterized by the combined presence of urea and Cd, and their specific concentrations. It has been reported that rice plants supplied with NH_4^+-N have comparatively lower oxidative damage and lower yield reduction under Cd stress than plants supplied with NO_3^--N (Jalloh et al., 2009), suggesting that plant growth and physiology responded variably to Cd exposure following the exogenous N-fertilizer addition. Consequently, not only should independent Cd toxicity be tested under model conditions, but also the interactions between different Cd concentrations and urea in aquatic plants. Insights on the physiological and biochemical mechanisms underlying plant tolerance to the combined action of Cd and urea could enhance our understanding of metabolic plasticity and the ranges of plant Cd tolerance. The combined action of urea and Cd in aquatic plants remains inadequately investigated.

Cabomba caroliniana A. Gray is a submersed aquatic plant native to freshwaters of South and North America (Ørgaard, 1991). The species has become an aggressive invader worldwide (Wilson et al., 2007; Wang et al., 2010; McCracken et al., 2013). It can grow in freshwater lakes, ponds, rivers, and streams. Because of its rapid growth and high biomass production, *C. caroliniana* is convenient plant material for ecotoxicological investigations. However, very few reports are available on Cd accumulation and toxicity, including the combined effects of Cd and urea in *C. caroliniana*. The objectives of this study were to investigate the effect of urea on Cd accumulation in *C. caroliniana* and the morphological and physiological response of *C. caroliniana* to the combined effects of urea and Cd, in order to address the questions above.

2. Materials and methods

2.1. Plant materials and experimental design

C. caroliniana plants with no traces of necrosis were obtained from an uncontaminated pond at Wuhan Botanical Garden, Chinese Academy of Sciences. For the experimental studies, plants were cleaned gently by hand to remove epiphytic algae. Healthy and uniform apical shoot segments with a length of 15–20 cm were chosen, and kept in glass aquariums containing 10% Hoagland's solution (Hoagland and Arnon, 1950; Huang et al., 2016). After one week of acclimation, plant materials were transferred to glass beakers. Various concentrations of Cd and urea were added to 10% Hoagland's solution to achieve the following treatments: 10 mg L^{-1} urea, 400 mg L^{-1} urea, 500 μmol L^{-1} Cd, 500 μmol L^{-1} Cd and 10 mg L^{-1} urea (Cd + 10 mg L^{-1} urea), and 500 μmol L^{-1} Cd and 400 mg L^{-1} urea (Cd + 400 mg L^{-1} urea). The concentrations of urea and Cd were chosen based on experimental trials. Preliminary experiments revealed that 400 mg L^{-1} urea had adverse effects on *C. caroliniana*. Considering the potential toxicity mechanism - oxidative damage induced by 400 mg L^{-1} urea - we used exogenous 5 mmol L^{-1} AsA to pretreat 400 mg L^{-1} urea exposed plants for 2 h, labelled 400 mg L^{-1} urea and 5 mmol L^{-1} AsA treatments (400 mg L^{-1} urea + AsA). AsA, a major primary antioxidant synthesized in the inner membrane of the mitochondria, reacts chemically with $^1O_2$, $O_2^{\cdot-}$, HO^- and thiyl radical (Layne et al., 1993) and acts as the natural substrate of many plant peroxidases (Foyer and Mulllineaux, 1998; Scebba et al., 2001). Thus, we could retroactively speculate the potential toxicity mechanism of urea from the results of the exogenous AsA application in response to *C. caroliniana* exposure to high concentrations of urea. The plants with no treatments were used as a control. Cd was applied as $CdSO_4$ and AsA solution was freshly prepared before treatments. All beakers were placed randomly in the growth chamber. The plants were cultured with a 12-h light/12-h dark cycle, at 25 ± 1 °C, and the photosynthetic active irradiation was approximately 125 ± 5 μmol photon m^{-2} s^{-1}. After 4 days of exposure, plants were harvested, washed with distilled water, and used in the measurement of various parameters.

2.2. Morphology and structure of the leaves

Leaves in the 3rd to 5th leaf storeys were sampled from shoot apices of *C. caroliniana* plants. Measurements of vegetative morphological characters were obtained by inspecting the leaves under a Motic BA310 Met light microscope.

2.3. Photosynthetic pigment, chlorophyll fluorescence measurements, oxygen exchange and Rubisco activity

The photosynthetic pigments were extracted from leaves with 95% ethanol at 4 °C in the dark for 24 h. Chlorophyll *a* (Chl *a*), Chlorophyll *b* (Chl *b*) and carotenoids (Car) were determined spectrophotometrically at 470, 648, and 663 nm (TU-1810PC, Purkinje General, China). The chlorophyll and carotenoid contents were calculated as described by Lichtenthaler (1987).

Parameters of chlorophyll variable fluorescence were determined using a pulse-modulated fluorometer PAM 2100 (Walz, Germany). Prior to measurements, the leaves were kept in darkness for 15 min to minimize fluorescence quenching. The measuring procedures can be found in Huang et al. (2016). The parameter F_v/F_m was used as an indicator of the maximal quantum yield of photosystem II (PSII) photochemistry (Kitajima and Butler, 1975). The effective quantum yield of PSII photochemistry (Rohacek, 2002) and the photochemical quenching coefficient (qP) (Schreiber et al., 1986) were also calculated.

After 4 days of treatment, net photosynthetic oxygen release and respiratory oxygen uptake were examined according to the method described by Shao et al. (2017), with an optical oxygen electrode (Unisense OX-13298) and a Unisense microsensor multimeter (Version 2.01). The chamber was placed in a homothermal water bath at 25 °C and illuminated by fluorescent tubes that provided a photon irradiance of 125 μmol m^{-2} s^{-1}. Rates of oxygen increase were recorded for 5–10 min. At the end of measuring photosynthesis, the chamber was placed in the dark and the decline in oxygen concentration monitored for about 10 min.