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Accumulation and acute toxicity of silver in *Potamogeton crispus* L.

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ABSTRACT

In the present study, *Potamogeton crispus* L. plants exposed to various concentrations of silver (Ag) (5, 10, 15, and 20 μM) for 5 d were investigated to determine the accumulating potential of Ag and its influence on nutrient elements, chlorophyll pigments and fluorescence, various antioxidant enzymes and compounds, adenosine triphosphate (ATP), protein content and ultrastructure. The accumulation of Ag was found to increase in a concentration dependent manner with a maximum of 29.3 $\mu g\,g^{-1}$ at 20 μM. The nutrient elements (except Ca), photosynthetic pigments, chlorophyll *a* fluorescence parameters (Fo, Fv, Fv/Fm, Fv/Fo), malondialdehyde (MDA), ATP, peroxidase (POD) activity, ascorbate (AsA), reduced glutathione (GSH) and protein contents decreased significantly as concentration of Ag augmented. In contrast, an induction in SOD activity was recorded, while an initial rise in Ca content and CAT activity was followed by subsequent decline. Morphological symptoms of senescence phenomena such as chlorosis and damage of chloroplasts and mitochondria were observed even at the lowest concentration of Ag, which suggested that Ag hastened the senescence of the tested plants. The loss of nutrients and chlorophyll content and damage of chloroplasts were associated with disturbances in photosynthetic capacity as indicated by the quenching of chlorophyll *a* fluorescence. Decreased chlorophyll and protein contents suggest oxidative stress induced by Ag. In addition, both the reduction of ATP and the damage to the ultrastructure of organelles were indicative of general disarray in the cellular functions exerted by Ag.

1. Introduction

Heavy metals are common pollutants in aquatic environment. The aquatic ecosystems are sensitive to pollutants due to the presence of relatively small biomass in a variety of trophic levels, which may lead to accumulation of heavy metals. Hence, aquatic plants are often the first link in relation to metal contents of aquatic environments [1]. Recently, several of the submerged, emergent and free-floating aquatic species have been identified as potential accumulators or biomonitors of heavy metals. These plants could play a role in biomonitoring studies and serve as a useful phytoremediation technology to restore water quality [2–5]. Excess heavy metals also caused a number of toxic symptoms which were always associated with visible injuries in aquatic plants, e.g. growth retardation, inhibition of photosynthesis and enzymes, oxidative stress and ultrastructural damage [3,6–11].

Silver is a non-essential heavy metal for any living organism, which enters the aquatic environment from natural and anthropogenic sources (photographic processing effluents, sewage sludge, biocidal and other applications). It is known that Ag ions interact metabolically with Cu and Se and replace H_2 from the sulfhydryl groups of the photosynthetic enzymes (such as Rubisco), changing their structure and inactivating them. Ag also forms complexes with amino acids, pyrimidines, purines and nucleotides, as well as with their corresponding macromolecular forms, suggesting its potential to be either highly toxic or easily inactivated by the plant [12]. To date, the impacts of Ag on freshwater ecosystems have achieved more and more attention [13–16]. However, there are very few toxicological data dealing with hydrophytes.

Potamogeton crispus L. (Potamogetonaceae), a rooted submerged plant, grows in freshwater lakes, ponds, rivers, and streams all over the world. It is a fast growing plant, which produces high biomass and has shown potential to accumulate considerable amounts of Cu, Pb, Mn, Ni, Zn [2], Hg [17] and Cd [18]. To our knowledge, few reports are available on the accumulation and toxicity of heavy metals in *P. crispus* [9–11,17–18] and no work has so far been carried out to study Ag-induced metabolic changes therein. In the present investigation, the influence of Ag bioaccumulation on several physiological and biochemical parameters in *P. crispus*, such as photosynthetic pigment and chlorophyll *a* fluorescence, nutrient status, antioxidants, ATP, protein and ultrastructure is studied in detail. This study will be helpful in elucidating the key mechanisms that lead to acute toxicity of Ag in freshwater macrophytes.

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2. Materials and methods

2.1. Plant material and Ag treatment of plants

The *P. crispus* plant was collected from unpolluted bodies of freshwater and acclimated for more than 6 months in large hydroponic tubes, subsequently, the growing shoots were cut off from the mother plant, washed thrice with running tap water and maintained under laboratory conditions in aquaria with 1/10 Hoagland solution at light irradiance of 114 $\mu mol\,m^{-2}\,s^{-1}$, photoperiod of 14 h, and temperature of 25/20 °C (day/night) [11].

After 2 weeks, the *P. crispus* plants with approximately the same height and weight were transferred to glass beakers. Various concentrations levels (i.e. 0, 5, 10, 15, and 20 μM) of Ag were supplied to the plants as $AgNO_3$ in 2 l 1/10 Hoagland nutrient medium for 5 d. Beakers were then placed in growth chamber under the conditions mentioned above. Ag concentration higher than 20 μM and the duration of the experiment longer than 5 d were found to be lethal to the plants. All solutions were refreshed every 2 d, and all experiments were performed in triplicate.

2.2. Elemental analysis

At the end of 5 d treatment, both control and Ag-exposed plants were washed thoroughly with 10 mM EDTA solution at 4 °C for 30 min under stirring, followed by double distilled water to remove adsorbed metal as well as nutrient ions from the surface of leaves. The content of Ag and nutrient elements was analyzed by Inductively Coupled Plasma Atomic Emission Spectrometry (Leeman labs, Prodigy, USA) after wet-digesting in concentrated HNO_3.

2.3. Photosynthetic pigment assay

Chlorophylls and carotenoids content (~0.4 g fresh samples) was extracted with 80% acetone in a mortar together with clean Fontainebleau sand and absorbances (A) at 470, 647 and 663 nm recorded on a spectrophotometer (Thermo GENESYS 10). The contents of Chl *a*, Chl *b* and carotenoids were determined according to Lichtenhaler [19].

2.4. Measurements of chlorophyll a fluorescence

Six leaves from control and Ag-treated plants were selected to measure chlorophyll *a* fluorescence parameters. Dark adaptation period for all the measurements was 20 min, and chlorophyll *a* fluorescence was determined using a portable fluorescence spectrometer Handy PEA (Hansatech Instruments, Norfolk, UK). The following parameters were measured: (1) Fo, initial/minimal fluorescence; (2) Fm, the maximal fluorescence; (3) Fv, variable fluorescence; (4) Fv/Fo, the maximum primary yield of photochemistry of photosystem II (PS II); (5) Fv/Fm, the maximum quantum yield of PS II.

2.5. Lipid peroxidation

The MDA content of leaves was determined using the thiobarbituric acid (TBA) method [20]. Plant tissue (0.5 g) was homogenized with 10 ml 10% (w/v) TCA. The homogenate was centrifuged at 10,000 × *g* for 10 min. To 2 ml of the aliquot of the supernatant, 2 ml of 10% TCA containing 0.5% TBA was added. The mixture was incubated at 95 °C for 30 min and then cooled quickly in an ice-bath. The contents were centrifuged at 10,000 × *g* for 15 min and the absorbance of the supernatant was measured at 532 nm and corrected for nonspecific absorbance at 600 nm. The concentration of MDA was calculated by using an extinction coefficient of 155 $mM^{-1}\,cm^{-1}$ and expressed as nmol g^{-1} fresh weight.

2.6. Activities of antioxidative enzymes

2.6.1. Extraction of enzymes

After the treatment, plant material (1 g) was put in a pre-cooled mortar, in which 0.05 M PBS buffer was added. After a grinding in ice-bath, the solid phase was centrifuged at 12,000 × *g* for 20 min at 4 °C. Supernatant was used to measure the activities of enzymes. The protein content in the supernatant was estimated according to Bradford [21].

2.6.2. Superoxide dismutase (SOD) assay

The activity of SOD was assayed by the method of Stewert and Bewley [22] by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture (3 ml) consisted of 50 mM phosphate buffer (pH 7.8), 130 mM methionine, 750 μM NBT, 20 μM riboflavin, 0.1 mM EDTA-Na_2 and a suitable aliquot of enzyme extract. After mixing, the test tubes were shaken and illuminated for 20 min at light irradiance of 4000 lx. The temperature was maintained at 25 °C. A tube containing protein kept in dark served as the blank while a tube kept in light without enzyme served as the control. The absorbance of the solution was taken at 560 nm. The activity of SOD was measured by subtracting NBT reduction in light with protein from NBT reduction in light without protein. One unit of the activity was defined as the amount of enzyme required to inhibit 50% of the initial reduction of NBT under light.

2.6.3. Peroxidase (POD) assay

The POD activity was determined with guaiacol [23] as the substrate in a total volume of 3 ml. In the presence of H_2O_2, POD catalyzed the transformation of guaiacol to tetraguaiacol (brown product). The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 6.1), 1% guaiacol, 0.4% H_2O_2 and enzyme extract. Enzyme extract (20 μl) was added to 3 ml reaction mixture. Increase in the absorbance due to oxidation of guaiacol was monitored at 470 nm (extinction coefficient 25.5 $mM^{-1}\,cm^{-1}$) at 10 s intervals up to 3 min using Thermo GENESYS 10 spectrophotometer. Enzyme activity was calculated by the increase in absorbance at 470 nm $min^{-1}\,g^{-1}$ fresh weight at 25 ± 2 °C.

2.6.4. Catalase (CAT) assay

CAT activity was measured at 405 nm by an assay of hydrogen peroxide based on the formation of its stable complex with ammonium molybdate [24]. One unit of CAT activity was defined as the decomposition of 1 μmol of hydrogen peroxide per minute.

2.7. Ascorbate (AsA) and reduced glutathione (GSH) determination

To determine the contents of AsA and GSH, the fresh leaves (0.5 g) were homogenized in ice-cold 5% (w/v) TCA and then centrifuged at 10,000 × *g* for 20 min at 4 °C. AsA was determined according to the modified procedure by Law et al. [25]. To measure total AsA, the supernatant was initially treated with dithiothreietol (which reduces dehydroascorbate to ascorbate): 0.2 ml of this was added to 0.5 ml of 150 mM phosphate buffer (pH 7.4) containing 5 mM EDTA and 0.1 ml of 0.5 mM *N*-ethylmaleimide. After adding 0.4 ml of 10% tricholoroacetic acid, 0.4 ml of 44% orthophosphoric acid, 0.4 ml of 4% dipyridyl in 70% ethanol and 0.2 ml of 3% ferric chloride, the mixture was incubated at 40 °C for 40 min. The color developed was read at 525 nm and the result was expressed as AsA content in the tissue (mg g^{-1} fresh weight). GSH content was determined spectrophotometrically at 412 nm by the method of Anderson [26], after precipitation with 0.1 M HCl, using GSH reductase, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB) and NADPH. The level of GSH was expressed as mg g^{-1} fresh weight.