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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.

Table 1
Effects of silver (Ag) supply on Ag and nutrient element contents in leaves of *P. crispus*. Data are means ±S.D., $n = 3$. Within a row, values followed by different letter are significant at $P < 0.05$.

Element content ($\mu g\, g^{-1}$)	Concentration (μM)				
	Control	5	10	15	20
Silver	ND	18.1 ± 0.3	24.9 ± 1.2	26.4 ± 1.02	29.3 ± 1.5
Potassium	$3410.4^{a} \pm 54.6$	$2920.8^{b} \pm 131.4$	$2550.3^{c} \pm 84.1$	$1920.7^{d} \pm 76.8$	$1740.9^{d} \pm 113.7$
Zinc	$33.8^{a} \pm 1.4$	$34.2^{a} \pm 1.1$	$25.4^{b} \pm 0.7$	$23.0^{c} \pm 0.3$	$23.5^{bc} \pm 0.8$
Copper	$0.8^{a} \pm 0.02$	$0.6^{b} \pm 0.02$	$0.4^{c} \pm 0.01$	$0.1^{d} \pm 0.0$	$0.1^{e} \pm 0.01$
Sodium	$633.9^{a} \pm 20.3$	$389.2^{b} \pm 4.7$	$381.5^{b} \pm 16.0$	$213.4^{c} \pm 3.4$	$164.5^{d} \pm 1.6$
Calcium	$1850.3^{a} \pm 79.6$	$2042.5^{b} \pm 24.5$	$1901.6^{a} \pm 68.4$	$1870.7^{a} \pm 39.3$	$1830.2^{a} \pm 76.9$
Iron	$34.3^{a} \pm 0.6$	$22.0^{b} \pm 1.8$	$25.7^{c} \pm 1.3$	$24.7^{c} \pm 1.2$	$25.6^{c} \pm 1.2$

2.8. ATP luminescent bioassay

The ATP content was detected by the method of Wang [27] using ATP (Sigma) as standard. 0.5 g fresh leaf sample was immersed into 10 ml Tris–HCl buffer (20 mmol l^{-1}, pH 7. 8) and boiled at 100 °C for 10 min immediately. After it was cooled at room temperature, the supernatant was used for estimation of ATP content. The reaction mixture consisted of 200 μl the extract and 800 μl luciferase, and the analysis of ATP content was based on luminous intensity recorded by FG-3000 luminometer (China). The ATP content was expressed as 10^{-10} M g^{-1} fresh weight.

2.9. Electron microscopy

Samples from leaves of control and Ag-treated plants of *P. crispus* were initially fixed for 2 h at 4 °C in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.3) and post-fixed in 1% aqueous osmium tetroxide for 2 h. Samples were dehydrated through a graded ethanol series and embedded in Epon 812 resin. Ultrathin sections of 70 nm thickness were cut using an Ultracut E ultramicrotome (Leica, Germany) with a diamond knife and then stained in 2% uranyl acetate and lead citrate. Afterwards, it was examined by a Hitachi H-600 transmission electron microscope.

2.10. Statistical analysis

The values in the text are mean values ±S.D. from at least three individual experiments. Regression analysis and correlation coefficients (r value) were performed using available statistical functions from the Microsoft Excel XP package. The significant differences between treatment and control samples were calculated using Student's t-test wherever applicable.

3. Results

3.1. Effects of Ag on the element contents in P. crispus

The bioaccumulation of Ag in the leaves of *P. crispus* was found to be increased in a concentration ($P < 0.05$) dependent manner (Table 1). Maximum accumulation of Ag was recorded in the leaves exposed to 20 μM Ag for 5 d.

The concentration of various nutrients changed markedly in response to outer Ag concentration compared with the control. The higher the accumulated Ag concentration, the greater the nutrient loss in *P. crispus* (Table 1) was. The correlation coefficients showed that the association between K, Zn, Cu, Na contents and Ag concentrations were found highly significant (r K = −0.9054, $P < 0.05$; r Zn = −0.8262, $P < 0.05$; r Cu = −0.9073, $P < 0.05$; r Na = −0.9428, $P < 0.01$). The change of Ca concentration was different from the concentration of K, Zn, Cu and Na, it reached peak value at 5 μM Ag, increasing by 10.38%. However, as the concentration of Ag continued to rise, it began to decline. With Fe content, it diminished by 35.86% at 5 μM Ag and about 26.14% at higher Ag concentrations, respectively.

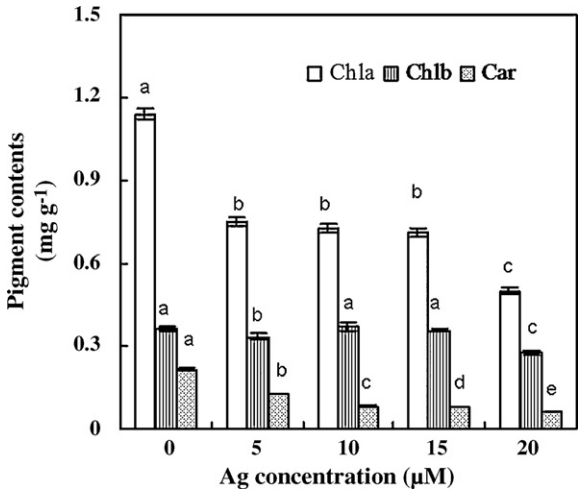

Fig. 1. The effect of silver (Ag) on the content of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and carotenoids (Car) in *P. crispus*. Values represent means ± S.D. ($n = 3$). Values designated over the bars in different letters are significantly different at $P < 0.05$.

3.2. Effects of Ag on pigment contents in P. crispus

The distinct decline in the chlorophyll *a* ($P < 0.05$), chlorophyll *b* and carotenoids (Car) ($P < 0.05$) contents was observed with increasing level of metal in nutrition medium (Fig. 1). Results indicated that the chlorophyll *a*, *b* and Car contents fell by 34.09%, 8.62% and 41.83% when the concentration of Ag was 5 μM. The maximum loss was 56.21%, 24.54% and 70.72% at 20 μM Ag as compared with control, respectively. Fig. 1 also showed that Chl *b* content decreased slower than Chl *a* and Car.

3.3. Effects of Ag on chlorophyll a fluorescence in P. crispus

Response of the fluorescence parameters of *P. crispus* to Ag stress was monitored after 5 d of metal treatment. It was noted that there was a progressive quenching of the initial fluorescence (Fo) ($P < 0.05$), maximal fluorescence (Fm) ($P < 0.01$), variable fluorescence (Fv) ($P < 0.01$), maximum primary yield of photochemistry of photosystem II (Fv/Fo) ($P < 0.01$) and maximum quantum yield of photosystem II (Fv/Fm) ($P < 0.05$) as a function of Ag concentration (Table 2).

3.4. Effects of Ag on lipid peroxidation in P. crispus

Generally, formation of malondialdehyde (MDA) is considered as a measure of lipid peroxidation in the plant. However, in our study, the correlation between the level of MDA and Ag concentrations is highly significant when using that of the correlation in the control as the null hypothesis ($P < 0.01$) (Fig. 2).

Table 2
Effects of silver (Ag) supply on Chl *a* fluorescence parameters. Data are means ± S.D. ($n = 6$). Within a row, values followed by different letter are significant at $P < 0.05$.

Parameter	Concentration (μM)				
	Control	5	10	15	20
Fo	$191.7^{a} \pm 26.6$	$164.5^{a} \pm 19.4$	$125.2^{b} \pm 11.7$	$123.3^{b} \pm 5.7$	$105.6^{c} \pm 8.8$
Fm	$1065.3^{a} \pm 147.8$	$841.0^{b} \pm 112.9$	$170.4^{c} \pm 59.7$	$133.7^{c} \pm 10.0$	$113.7^{d} \pm 15.1$
Fv	$873.7^{a} \pm 125.5$	$676.5^{b} \pm 105.9$	$38.5^{c} \pm 8.7$	$4.2^{c} \pm 2.1$	$3.8^{c} \pm 1.6$
Fv/Fo	$4.6^{a} \pm 0.4$	$4.1^{a} \pm 0.7$	$0.4^{b} \pm 0.1$	$0.05^{c} \pm 0.01$	$0.04^{bc} \pm 0.02$
Fv/Fm	$0.8^{a} \pm 0.01$	$0.8^{a} \pm 0.03$	$0.2^{b} \pm 0.02$	$0.04^{b} \pm 0.01$	$0.03^{b} \pm 0.02$

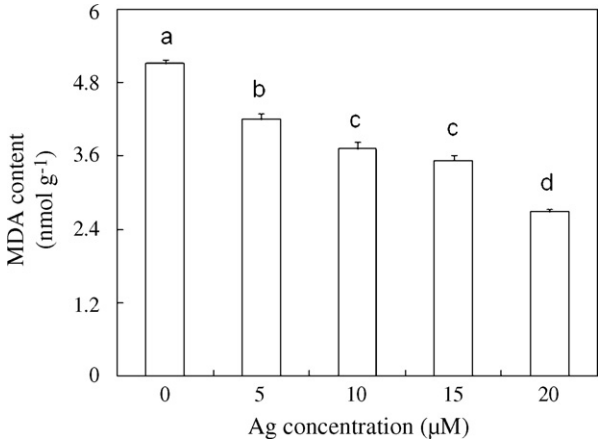

Fig. 2. MDA content in leaves of *P. crispus* in response to various levels of Ag stress. Data are means ± S.D. ($n = 3$). Values designated over the bars in different letters are significantly different at $P < 0.05$.

3.5. Effects of Ag on protein content in P. crispus

It could be seen from Fig. 3 that protein content dropped down abruptly ($P < 0.01$) as Ag concentrations increased. The maximal loss in protein content was recorded as 72.53% in leaves of the plant exposed to 20 μM Ag.

3.6. Effects of Ag on activities of antioxidant enzymes in P. crispus

Analysis of antioxidant enzymes (SOD, POD and CAT) showed considerable different changes in their activity upon supply of Ag (Table 3). When compared to controls, both SOD and CAT showed almost similar response. Activity of the former was significantly enhanced ($P < 0.01$) in the leaves of Ag-treated *P. crispus* at all

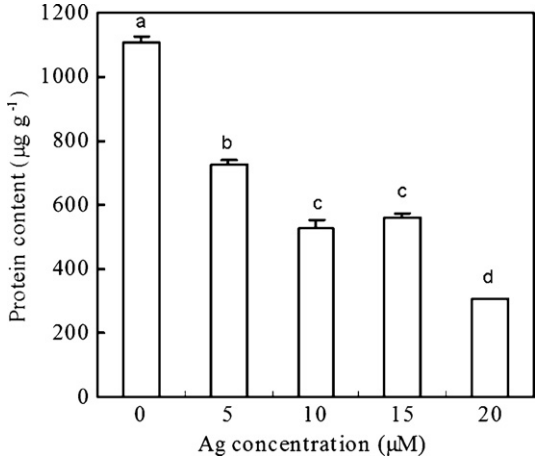

Fig. 3. Protein content in leaves of *P. crispus* in response to various levels of Ag stress. Data are means ± S.D. ($n = 3$). Values designated over the bars in different letters are significantly different at $P < 0.05$.

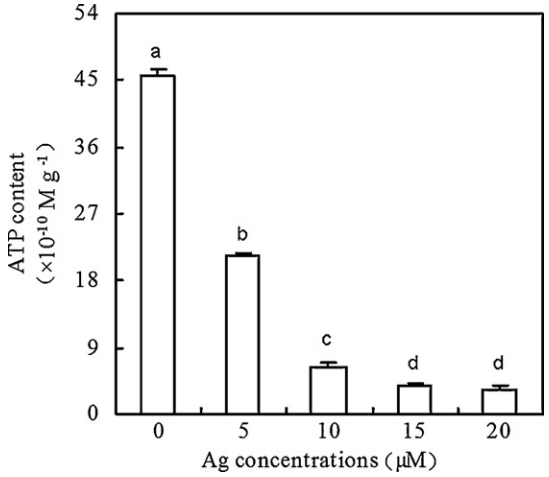

Fig. 4. ATP content in leaves of *P. crispus* in response to various levels of Ag stress. Data are means ± S.D. ($n = 6$). Values designated over the bars in different letters are significantly different at $P < 0.05$.

the concentrations, and the maximum level of the latter being 102.44 U g^{-1} at 5 μM Ag (42.11% higher than controls). Despite the decrease in CAT activity at higher concentrations, it remained higher than controls. In contrast to SOD and CAT activity, a significant decline ($P < 0.05$) was observed in the POD activity in leaves of Ag-treated *P. crispus* in a dose dependent manner.

3.7. Effects of Ag on ascorbate (AsA) and reduced glutathione (GSH) in P. crispus

The levels of both AsA and GSH in Ag-treated plants showed gradual ($P < 0.01$) decrease at all concentrations (Table 3). The maximal reduction (18.97% for AsA and 24.19% for GSH) was detected under 20 μM Ag treatment.

3.8. Effects of Ag on ATP content in P. crispus

The Ag treatment resulted in a drastic decline of ATP level ($P < 0.01$) in leaves of *P. crispus* (Fig. 4) with increasing Ag in solution. This reduction reached 92.57% of the control under the highest Ag concentration and the inhibition showed a clear dependence on Ag concentration ($EC_{50} = 2.3$ μM).

3.9. Effects of Ag on ultrastructure of leaf cells in P. crispus

Under normal conditions, chloroplasts in the control plants are lens-shaped, with orderly arrangement of grana and stroma thylakoids and one or two grains of starch (Fig. 5A). Mitochondria appear to be round, oval and oblong, with distinct tubular cristae (Fig. 5E). By contrast, exposure to Ag had pronounced effects on ultrastructure of chloroplasts and mitochondria. In the cell of fronds treated with 5 μM Ag, it could be clearly observed that the chloroplasts seemed to be global and breakage occurred on the outer membrane (Fig. 5B). The electron density of some region deceased

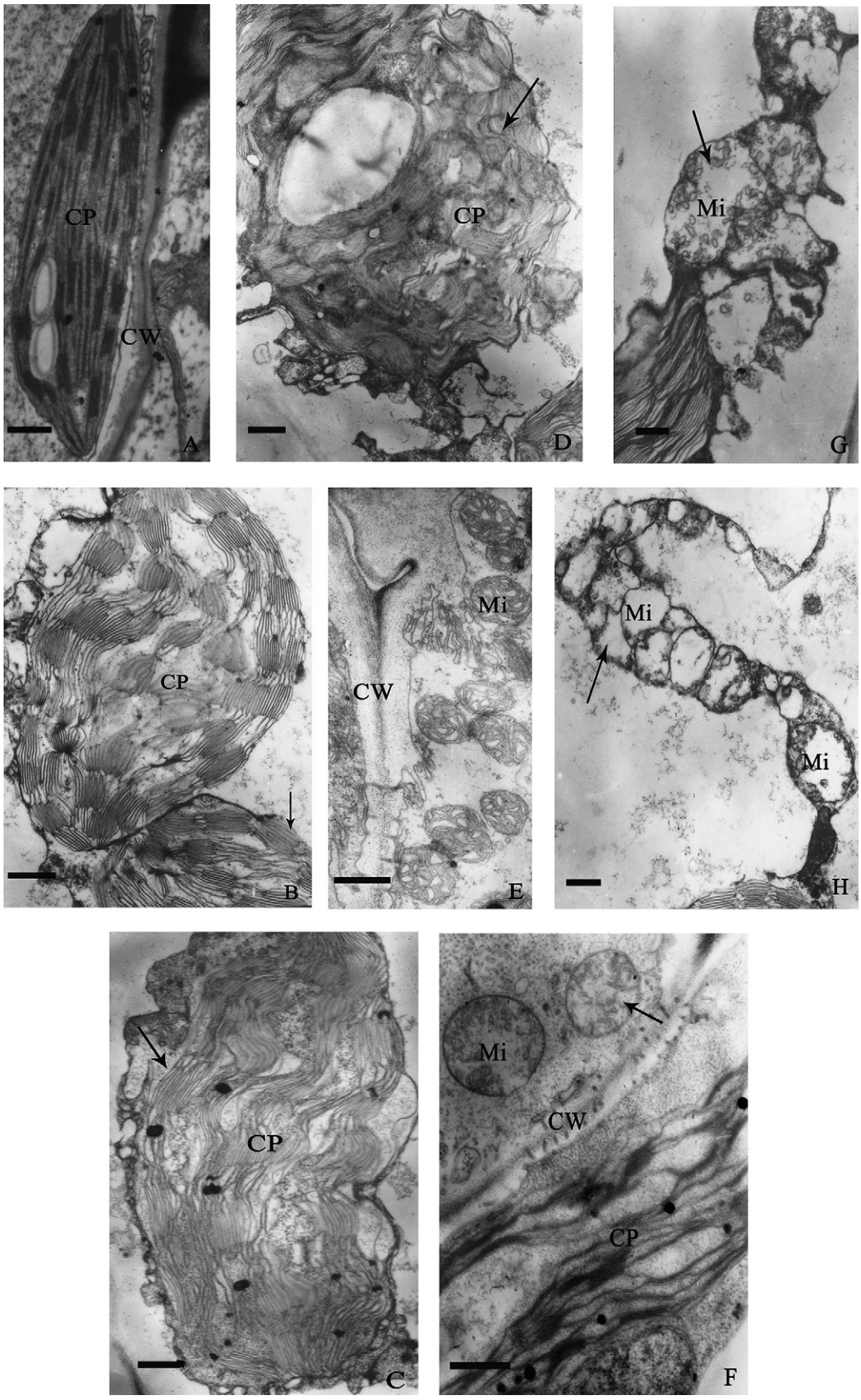

Fig. 5. (A) Chloroplast (CP) with orderly arrangement of grana and stroma thylakoids and one or two grains of starch in control leaf cells (Bar = 50 μm). (B) Global chloroplasts and breakage occurred on the outer membrane in 5 μM Ag-treated fronds (Bar = 50 μm). (C) Outer membrane of chloroplast was damaged in 15 μM Ag-treated fronds (Bar = 50 μm). (D) Disintegrated chloroplasts in 20 μM Ag-treated fronds (Bar = 50 μm). (E) Round, oval and oblong mitochondria (Mi) with distinct tubular cristae in normal leaf cells (Bar = 50 μm). (F) Electron density of some region deceased in mitochondria in the cell of fronds treated with 5 μM Ag (Bar = 50 μm). (G) Leaf cells treated with 10 μM Ag, showing mitochondria (Bar = 50 μm). (H) Vacuolar mitochondria in 15 μM Ag-treated leaf cells (Bar = 50 μm).

Table 3
Effects of silver (Ag) supply on levels of antioxidants. Data are means ± S.D., $n = 3$. Within a row, values followed by different letter are significant at $P < 0.05$.

Levels of antioxidant enzymes and antioxidants	concentration (μM)				
	Control	5	10	15	20
SOD ($U\,g^{-1}$)	$499.7^{a} \pm 16.0$	$557.6^{a} \pm 34.6$	$566.5^{b} \pm 29.5$	$609.5^{c} \pm 18.9$	$619.8^{d} \pm 9.3$
POD ($\Delta OD_{470\,nm} min^{-1} g^{-1}$)	$899.1^{a} \pm 37.8$	$695.3^{d} \pm 9.0$	$588.7^{c} \pm 18.8$	$550.6^{c} \pm 39.6$	$550.6^{c} \pm 38.5$
CAT ($U\,g^{-1}$)	$96.1^{a} \pm 3.1$	$136.6^{d} \pm 5.9$	$134.1^{b} \pm 7.0$	$109.4^{c} \pm 2.3$	$102.4^{ac} \pm 3.9$
AsA ($mg\,g^{-1}$)	$3.0^{a} \pm 0.1$	$2.9^{a} \pm 0.1$	$2.5^{b} \pm 0.03$	$2.4^{b} \pm 0.1$	$2.4^{b} \pm 0.03$
GSH ($mg\,g^{-1}$)	$8.3^{a} \pm 0.1$	$7.6^{b} \pm 0.2$	$6.7^{c} \pm 0.1$	$6.5^{c} \pm 0.2$	$6.3^{d} \pm 0.2$

in mitochondria, some cristae dilated a little and the outer membranes of some mitochondria broke down (Fig. 5F and G). The dose of 15 μM Ag damaged most outer membrane of chloroplast (Fig. 5C) and the whole vacuolar mitochondria were also found (Fig. 5H). With the further increase in Ag concentration, the thylakoid grana were damaged and chloroplasts were disintegrated (Fig. 5D).

4. Discussion

The accumulation of metals in aquatic macrophytes was often accompanied by an induction of a variety of cellular changes, some of which directly contribute to metal tolerance capacity of the plants [3,8] and some sensitive metabolic parameters (with significant stimulatory or inhibitory effects) could be used as biomonitors of heavy metals [6]. In the present study, as non-essential trace metal, Ag accumulation in *P. crispus* also resulted in considerable physiological, biochemical and ultrastructural changes.

Different from the mesophytes, *P. crispus* a completely submerged macrophyte, absorbs mineral elements directly from the aquatic body through its wide leaf surfaces the same way as other submerged species (*Elodea canadensis*) [28]. In previous studies, increase in Ag and other toxic metals in the culture medium resulted in significant prevention in the uptake of various nutrients which was deleterious to the plant [6,12]. A similar result was also observed in the present study in relation to content of K, Na, Cu, Zn and Fe in Ag-treated *P. crispus*. The results herein revealed that Ag disturbed the intrinsic balance of nutrient elements in cells. This nutrient deficiency might result from the reduced availability of energy (ATP) on which the functioning of membrane transport systems depended (Fig. 4) and the metal-induced disorder in the cell metabolism. Especially, the decline in Fe uptake was a typical example of cell metabolic disorders produced by the heavy metals [29].

Chlorophyll content and chlorophyll fluorescence are used to highlight stress due to a single environmental factor or to a combination of different environmental factors, but they also constitute potential biomarkers of anthropogenic stress and biotic stress [30]. A loss in the levels of chl *a*, *b*, and carotenoids was obvious in aquatic plants exposed to increasing concentrations of heavy metals (Hg and Cd) [1,3,6,18]. In the present study, pigment content in leaves of *P. crispus* also reduced as Ag exposure increased from 5 to 20 μM. Ag has been reported to interfere with chlorophyll biosynthesis and/or breakdown as other metals [3,6,18,31]. Fe and Mg deficiency with Ag was the reason for the reduced chlorophyll formation [12]. Change in chloroplast ultrastructure induced by elevated Ag was another important reason explaining the decrease of chlorophyll content. This was also evident from our present study as well as earlier investigations [10,11], wherein Pb and Cd treatment resulted in a significant loss in chlorophyll content and ultrastructural damage of chloroplast in the same plant species, respectively. On the other hand, the slower degradation of Chl *b* suggested involvement of photoxidative mechanisms in Ag-induced toxicity [3,8,32]. It also indicated that Chl *a* and Car were more sensitive than Chl *b* to Ag stress. Decrease of these pigments implied direct reductions in photosynthetic activity, and hence reduced carbon fixation and possible effects at the whole plant level [33].

Chlorophyll fluorescence measurement was considered as a way to evaluate the biochemical and physiological state of plants [30]. In this study, on exposure to Ag, the significant decline of the maximum/potential efficiency of PS II (Fv/Fm) showed an impairment of the primary photochemical efficiency of the photosynthetic apparatus during the 5 d of the experiment. The same influence of Ag on the pigment content indicated that the reduced chlorophyll content was one of the reasons for the decreased photosynthetic efficiency. Inhibition of electron transport and enzymatic processes in the Calvin cycle by Ag has also been postulated by Ouzounidou [12]. In addition, the decreased Fv/Fo reflected the inactivation of chlorophyll associated with the reaction centers of PS II [12] (Table 2). The reduction of Fv/Fm and Fv/Fo in Ag-treated fronds was mainly resulted from the decrease of variable fluorescence (Fv) (Table 2) indicating Ag perturbation on the acceptor side of PS II [34]. Complete elimination of fluorescence signals (such as Fo, Fm, Fv, Fv/Fm and Fv/Fo) occurred under Ag stress indicating the extreme nature of the stress exposure, which could attribute to the membrane breakdown, thylakoid dysfunction and enzyme deactivation [35]. The loss of nutrients (Table 1) and chlorophyll pigment (Fig. 1), and the damage to chloroplasts (Fig. 5) induced by Ag also supported the overall disturbances in photosynthetic function as indicated by the chlorophyll *a* fluorescence.

A common feature of both abiotic and biotic stress is the generation of reactive oxygen species (ROS). The protective mechanisms adapted by the plants to scavenge free radicals and peroxides include several antioxidant enzymes (peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD)) and antioxidant compounds (glutathione (GSH), ascorbate and carotenoids) [36]. The antioxidant enzymes were important components in preventing the oxidative stress from the fact that the activity of one or more of these enzymes was generally increased in plants when exposed to toxic metals [1,3,9–11]. It is evident from our study that the protective enzymes of *P. crispus* lost their intrinsic balance and exhibited varied response to the Ag treatments. The increase in SOD and CAT activities in leaves of *P. crispus* detected following Ag treatment could be considered as a circumstantial evidence of enhanced production of free radicals. Apart from these active oxygen-scavenging enzymes, the levels of antioxidants like reduced GSH, AsA and carotenoids in Ag treated fronds were all lower than those in control, which would increase the susceptibility of plants to free radical damage [37]. As a matter of fact, Ag caused significant oxidative damage as evidenced by decreased chlorophyll and protein content [1]. The results herein suggested that the tolerance capacity of the plants to the toxic metals depend on the equilibrium between the production of ROS and the quenching activity of the antioxidants. The apolexis and death of plants in the stress of Ag might be due to oxidative stress which exceeded the cellular antioxidant defense capacity. Moreover, ultrastructural alterations, such as swelling of mitochondrial cristae and thylakoids and disintegrated chloroplasts, were considered as important signals of heavy metal induced senescence [28] and finally caused the disturbance or inhibition of cellular functions [38].

Dhir et al. [39] reported that the level of MDA (a major cytotoxic product of lipid peroxidation and widely accepted to be an indicator of free radical/TOS production) in hydrophytes (*Hydrilla*, *Wolffia* and *Ceratophyllum*) grown under control conditions was relatively higher than those exposed to Cd^{2+} pollution. A similar trend of decline was also seen in Cu-treated *Hydrilla verticillata* [40] and Hg-treated *Bacopa monnieri* [41] in the case of MDA. This was as the result of the decrease in the total polyunsaturated fatty acids (PUFA) which was prone to peroxidative damage [40,42]. These results were in accordance with our present findings on *P. crispus* which showed a concentration-dependent decrease in MDA content and demonstrated that *P. crispus* could not exhibit lipid peroxidation under Ag stress like other plant species [1]. It suggested that different adaptive mechanisms existed in *P. crispus* to tackle the acute stress of Ag. Being the precursor for phytochelatins (PCs), GSH was consumed by the ascorbate-GSH pathway, by which H_2O_2 was scavenged and PCs were catalyzed by phytochelatin synthetase. Therefore, the decrease in the GSH concentrations indicated the possibility of PCs synthesis in *P. crispus* from the fact that GSH was depleted during PCs synthesis, in spite of no evidence for the induction of PCs was provided in the present investigation. In fact, for metals other than Cd and Cu, PCs were induced to varying levels by a wide range of metal ions and Ag was considered as the most effective one [43]. Extensive experimentation is required to elucidate the induction of PCs and their roles in counter balancing the acute toxic doses of Ag in *P. crispus*.

Heavy metals are known to severely affect the plant ATP content. The decrease in the ATP amount is a characteristic response to the toxicity of heavy metals [44,45] and an expected corollary to general disarrays in the cellular functions [46]. In our experiment, Ag exposure resulted in a significant reduction in ATP content in leaves of *P. crispus* which possibly owing to the need for development of a detoxification system (metallothionein synthesis, energy-driven efflux pumps, etc.) [45]. ATP content depended on various enzyme activities involved in either ATP synthesis or degradation, however, Ag inhibited one or more of these enzyme activities (such as ATPase) [12] and thus affected ATP content indirectly. Moreover, significant impairment in ultrastructure of mitochondria and chloroplasts in Ag-treated leaf cells (Fig. 5) would inevitably affect the oxidative- and photo-phosphorylaton and the consequent reduction in ATP content.

5. Conclusions

The present study demonstrated the physiological, biochemical and ultrastructural impacts of Ag on leaves of *P. crispus*. Reduction in almost all the parameters (except SOD, Ca and CAT) was observed at all Ag concentrations. The uptake of various nutrients was prevented significantly (except Ca) with the rise of Ag concentration accumulated in leaves of *P. crispus*. The chlorophyll and carotenoid decrease at low Ag concentration was indicative of damage at the chloroplast level. Changes in scavenging enzyme activities (SOD, POD and CAT), antioxidative compounds (AsA and GSH) and fine structure provided precise information on the induction of oxidative stress and were also evidenced by decreased chlorophyll and protein contents. Ag-induced severe ultrastructural changes in leaf cells of *P. crispus* and the severity of damage enhanced with increasing Ag concentrations. The observed ultrastructural impairment (chloroplast and mitochondrion alterations) confirmed that chloroplasts and mitochondria were major targets in the mechanism of Ag toxicity, which was responsible for disarrays in cellular functions and senescence of leaves. Furthermore, the decrease in Fv/Fm and Fv/Fo pointed to photoinhibition and inactivation of chlorophyll associated with the PSII reaction center. The decrease of MDA content demonstrated that *P. crispus* could not exhibit lipid peroxidation under Ag stress. It was suggestive of different adaptive mechanisms existing in the tested plant. Further research is necessary to clarify the possible involvement of PCs in Ag detoxification in *P. crispus* since the GSH depressed significantly at all the Ag treatment.

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