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Antioxidative Response of *Hydrilla verticillata* (L.f.) Royle under Short Term Exposure to Mercuric Chloride

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Authors' contributions

This work was carried out in collaboration between all authors. Author SP carried out the experimental work, author SM compiled the data and performed the statistical tests and author SKD conceptualized the research frame work, designed and supervised the experiments and drafted the manuscript. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Hydrilla verticillata (L.f.) Royle twigs were subjected to $HgCl_2$ (10, 50, 100 and 200 mg/L) for 3 h under light and dark conditions. There was significant loss in total chlorophyll, soluble protein and ascorbic acid contents in the twigs with increased Hg concentrations in the medium. Superoxide dismutase (SOD) activity increased in a concentration dependant manner indicating the increased protection against superoxide radical under Hg stress but at the same time the generation of higher amount of H_2O_2 as dismutation product was also favoured. At lower concentration, there was increase in catalase (CAT) activity but it declined significantly towards higher concentrations of Hg as a result the protection against H_2O_2 weakened. Even though there was increase in peroxidase (POX) activity, it could not be attributed to efficient H_2O_2 scavenging. Malondialdehyde (MDA) content in the tissues increased which suggested that there was imposition of oxidative stress due to Hg. The addition of antioxidant (ascorbate) to the medium reduced the toxic effects. The results suggest that the Hg induced oxidative stress was probably due to alterations in the activities of key antioxidative enzymes *viz* SOD, CAT and POX. Therefore, increased antioxidant efficiency could increase the tolerance of the plant to the metal and thereby it could be a better candidate for Hg bioaccumulation in polluted water bodies.

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1. INTRODUCTION

The use of heavy metals in different anthropogenic activities is an age old practice. Increased industrial and mining activities during the late 19^{th} and early 20^{th} century have augmented the environmental pollution due to heavy metals. Mercury (Hg), cadmium (Cd) and lead (Pb) are three such heavy metals which are known to be most toxic in the environment [1]. Mercury is a unique element which is liquid at room temperature and is 13.6 times heavier than water. This metal is relatively uncommon in the Earth's crust and from the processes like erosion, volcanic eruption and mining it is liberated. Most of the mercury in the environment is released by intentional anthropogenic activities such as mercury manufacture and disposal and unintentional activities including fossil fuel burning [2]. Mercury occurs in nature in different forms and the most common among them are metallic or elemental Hg, mercuric sulphide, methyl mercury and mercuric chloride. These species differ greatly in properties but all are toxic and there is no safe limit to mercury.

The rivers and estuaries receive huge amounts of sewage and industrial effluents which usually constitute the principal sources of aquatic pollution. In the aquatic bodies the different pollutants like heavy metals, radionuclides and organic chemicals initially become associated with suspended particulates and in due course of time, become deposited in the sediments. Therefore, when the primary sources of metal pollution are eliminated, the sediments may act as secondary sources and hence pose a potential long-term threat to the aquatic environment [3]. Due to their toxicity and bioaccumulation behaviour, the pollution of aquatic ecosystem by heavy metals has assumed serious concern. Among the different sediment bound metals in the aquatic environment, mercury in particular is of major ecogenotoxicological concern because it not only undergoes transformation to highly toxic methyl mercury but is also biomagnified during the course of its passage through the food chain. At cellular level, plants are known to exhibit different types of responses to mercury toxicity. Some of the possible mechanisms of its phytotoxicity include change of the membrane permeability, high affinity to react with the sulphydryl (SH) groups, affinity for reacting with phosphate groups and the replacement of essential ions and its ability to disrupt functions involving critical or nonprotected proteins [4,5]. According to Watson [6], most of the increases in environmental mercury occur in the less developed regions of the world which can be attributed to economic constraints and/or lack of proper environmental protection regulations. The maximum concentrations of mercury in the environment have been found near industrial sites such as chloralkali plants. For developing any strategy for the reduction of toxic effect of any pollutant, studies on the physiological response of the organism to that particular pollutant is highly essential. Antioxidative system in the aerobic organisms constitutes different antioxidative enzymes and low molecular weight antioxidants that neutralize the deleterious effects of reactive oxygen species (ROS) [7]. The ROS includes superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and singlet oxygen ($^1O_2$) which are known to generate during normal oxygen metabolism. However, their rate of generation is also augmented in different developmental and also under different stress situations [8,9] beyond the capacity of the endogenous antioxidative protective system to scavenge them off, creating a situation called oxidative stress. Thus the oxidative metabolism is a regulated process between the generation of ROS and their subsequent scavenging. Reports on the antioxidative efficiency of plants in aquatic environment under mercury stress are rare. Therefore, in this study the antioxidative response of plants under short term exposure to $HgCl_2$ has been investigated by assessing

the activities of antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) taking *Hydrilla verticillata* (L.f.) Royle, an aquatic macrophyte, as an experimental system. The level of lipid peroxidation in the tissues was measured and attempt for neutralizing Hg induced toxicity under such exposure was also done.

2. MATERIALS AND METHODS

2.1 Plant Material, Growth Condition and Stress Imposition

Fresh and healthy twigs of *Hydrilla verticillata* (L.f.) Royle were collected from local pond and were washed carefully with tap water to remove the particulate matters deposited on the plant surface, if any, before the metal treatment. Twenty ml of different concentrations of $HgCl_2$ (10, 50, 100 and 200 mg/L) were taken in test tubes and to each test tube; one twig was introduced in a manner so that the twig remained completely immersed in the solution. Distilled water was taken in another test tube as control. One such set was kept in dark and another set was kept under light. The exposure of the plant to Hg was done for 3 h and in all the treatments, the twigs were selected for uniformity of size. However, in all the cases the fresh weight of the twig was recorded before analysis and the amount of tissue taken for analysis are mentioned against the respective parameter. The light source was a bank of 40 W Phillips cool fluorescent tube lights supplemented with 40 W incandescent bulbs giving the photosynthetic photon flux density of approximately 90 µmol. $m^{-2}s^{-1}$. To study the effect of antioxidant (ascorbate) on neutralizing the toxic effects of Hg, two different concentrations of ascorbate (1 and 10 mM) were taken separately along with 50 mg/L $HgCl_2$ and exposure of the tissues to these combinations were done for 3 h, under light.

2.2 Extraction and Estimation of Total Chlorophyll

After 3 h of exposure to $HgCl_2$, the twigs were washed with water and total chlorophyll content was extracted by homogenizing the tissue (200 mg) with cold 80% acetone and the volume of the homogenate was made to 5 ml. Homogenates were then centrifuged at 1900 × *g* for 10 min and the absorbance of the supernatant was read at 645 and 663 nm in a spectrophotometer. Total chlorophyll content was calculated as described by Arnon [10].

2.3 Extraction and Estimation of Soluble Protein

Buffer soluble protein was precipitated by mixing 1 ml of enzyme supernatant (the preparation of which is mentioned below) with equal volume of 20% (v/v) trichloroacetic acid (TCA). The precipitates were then washed successively with cold 10% (v/v) TCA; ethyl alcohol; ethyl alcohol: chloroform (3:1, v/v); ethyl alcohol: ether (3:1, v/v) and finally with ether. The pellets were air dried and suspended with 2 ml of 0.3 N NaOH for 16 h at 37ºC. Then the samples were centrifuged and supernatants collected as protein extract and soluble protein content was estimated following the method of Lowry, et al. [11], taking bovine serum albumin as standard.

2.4 Extraction and Estimation of Ascorbic Acid

For extraction of ascorbic acid the twigs were washed properly and 200 mg of tissue was homogenized separately with 6% metaphosphoric acid and the volume was made to 5 ml. The homogenates were centrifuged at 1900 × *g* for 10 min and the supernatants were used after suitable dilutions, for the spectrophotometric estimation of ascorbic acid following the

method of Mitsui and Ohta [12]. The ascorbic acid content of the tissue extract was calculated by comparing the absorbance with a standard curve prepared by taking 0 to 176 µg of ascorbate per assay.

2.5 Extraction and Estimation of Malondialdehyde (MDA)

Malondialdehyde (MDA) content was estimated in the tissues exposed to $HgCl_2$ for measuring the lipid peroxidation level, following the method of Heath and Packer [13] taking thiobarbituric acid (TBA) as the reactive material. After 3 h of exposure, the twigs were properly washed and 200 mg of tissue in each case was homogenized with 5% (w/v) TCA and the volume of the homogenate was made to 1 ml. The entire homogenate (i.e., 1 ml) was mixed with 4 ml of TBA reagent (0.5% TBA in 20% TCA). The reaction mixtures were heated at 95ºC for 30 min in a water bath and then cooled quickly in an ice bath and centrifuged at 1900 × *g* for 10 min. The absorbance of the supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm and for absorbance at 532 nm of the correction blank. 1 ml of 5% (w/v) TCA was mixed with 4 ml of TBA reagent for reference blank and 1 ml of tissue homogenate was mixed with 4ml of 20% (w/v) TCA for correction blank. MDA content in the tissues was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} for malondialdehyde at 532 nm.

2.6 Preparation of Cell Free Extract and Assay of Enzyme Activities

For extraction of enzymes, the twigs (taking 200 mg of tissue in each case) were homogenized under ice-cold conditions in a mortar and pestle using sodium phosphate buffer of 0.05 M, pH 7.4 for superoxide dismutase (SOD) and 0.05 M, pH 7.5 for catalase (CAT) and guaiacol peroxidase (POX) and the volume of the homogenate was made to 5 ml. The homogenates were centrifuged at 17, 000 × *g* for 10 min at 0ºC and the resultant supernatants were passed through gel filtration columns (12 mm diameter and 50 mm long), packed with pre-soaked Sephadex G-25 fine. The eluted fractions responding to protein test were pooled out and used directly for enzyme assays, after suitable dilution. Superoxide dismutase (SOD; EC 1.15.1.1) was assayed by measuring the inhibition of superoxide-driven nitrite formation from hydroxylamine hydrochloride following the method of Das, *et al*. [14]. The SOD activity was computed from the value of V_o/V -1, where V_o is the absorbance of the control (without enzyme) and V is the absorbance of sample (with enzyme). Catalase (CAT; EC 1.11.1.6) activity was assayed by measuring the rate of decomposition of H_2O_2 in the reaction mixture at 240 nm following the method of Aebi [15] and was calculated using extinction coefficient of 40 $mM^{-1}cm^{-1}$ for H_2O_2 at 240 nm. Guaiacol peroxidase (POX; EC 1.11.1.7) was assayed by taking H_2O_2 and guaiacol as substrates as described by Kar and Feierabend [16]. The increase in the concentration of tetraguaiacol formation in the reaction mixture due to POX activity was recorded at 470 nm and POX activity was calculated using the extinction coefficient of 26.6 $mM^{-1}cm^{-1}$ for tetraguaiacol at 470 nm.

SOD activity is expressed in unit (U) and one unit is the amount that inhibits the superoxide driven nitrite formation from hydroxylamine hydrochloride by 50%, under the assay conditions. CAT and POX activities are expressed in katals, i.e., moles of substrate used up or product formed due to enzyme activity in the reaction mixture per second.

2.7 Data Presentation and Statistical Test

The results presented are the mean values of three independent experiments with three replicates in each time. Standard deviations are indicated as vertical bars. The significance of difference among the mean values, for a particular experimental condition (light or dark, not between light and dark) was determined following Least Significant Difference (LSD) test [17]. To indicate the significance of difference, the letters used for the "light conditions" are 'a', 'b', 'c', 'd' and 'e' and the same for "dark conditions" are 'f', 'g', 'h', 'i' and 'j'.

3. RESULTS

In this study, toxic symptom was visually noticed in the form of bleaching of the twigs, which was quite distinct at higher concentration (i.e., 200 mg/L), even within 3 h of exposure. The total chlorophyll loss was in concentration dependent manner both under light and dark conditions (Fig. 1A). The pigment loss was significant (P=0.05) even at lowest concentration of the metal tested, i.e., at 10 mg/L $HgCl_2$, in comparison to control twig and the loss was found to be more under light than the corresponding dark samples. At 200 mg/L of $HgCl_2$, bleaching in the sample kept under light was visually noticeable and the chlorophyll loss in this sample was about 65% of the control one. Therefore, all the analytical works were restricted within 200 mg/L of $HgCl_2$. It was found that at 50 mg/L $HgCl_2$, there was 40% loss in the total chlorophyll than the control tissues. But with the addition of ascorbate in the medium along with 50 mg/L $HgCl_2$, there was about 14% gain at 1 mM and 57% gain at 10 mM of ascorbate over the mercury induced loss of the pigments (Table 1). With 10 mM ascorbate, the total chlorophyll content of the twigs reached almost the level of control twigs.

Table 1. Effect of ascorbate in the restoration of Hg induced total chlorophyll loss in *Hydrilla verticillata* (L.f.) Royle twigs

Treatment	Total chlorophyll (mg. g FW^{-1})
Control (water)	$1.75^{a} \pm 0.157$
$HgCl_2$ (50 mg/L)	$1.05^{d} \pm 0.141$
$HgCl_2$ (50 mg/L) + Ascorbate (1 mM)	$1.196^{c} \pm 0.136$
$HgCl_2$ (50 mg/L) + Ascorbate (10 mM)	$1.65^{b} \pm 0.165$

The results are mean of three independent experiments with three replicates in each experiment. The mean values followed by different letters (in superscript) are statistically significant (P=0.05; LSD test).

There was also decrease in soluble protein content but it was not significant at lower concentration of the metal. In presence of light there was 42% and under dark there was 26% decrease in soluble protein content of the twigs in comparison to the respective control twigs (Fig. 1B). The ascorbic acid content of the twigs declined sharply with increase in Hg concentrations in the medium. In presence of light there was 71% and under dark there was 51% decrease in ascorbic acid content of the twigs in comparison to the same of the respective control twigs (Fig. 1C).

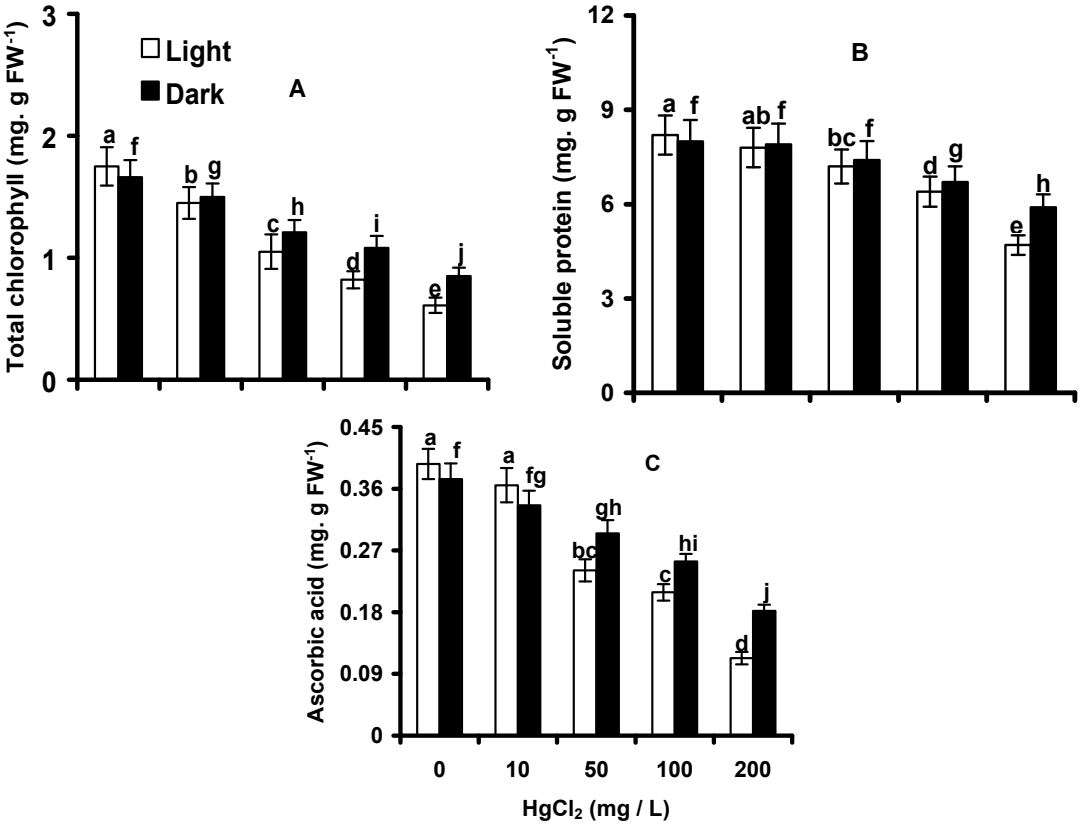

Fig. 1. Changes in (A) the total chlorophyll, (B) soluble protein and (C) ascorbic acid contents of *Hydrilla verticillata* (L.f.) Royle twigs exposed to $HgCl_2$ for 3 h under light and dark conditions

The results are mean, with standard deviation, of three independent experiments with three replicates in each time. The differences among the mean values, for a particular experimental condition (light or dark, not between light and dark), followed by the same letter are not statistically significant (P = 0.05; LSD test)

The activities of three important antioxidative enzymes *viz,* SOD, CAT and POX were assayed in this study. The activity of SOD was found to low in control twig but with increase in the metal concentration the activity increased significantly (Fig. 2A). At severe stress (i.e., 200 mg/L of $HgCl_2$), there was about 10 times increase in SOD activity in the sample kept under light in comparison to the control twig and 7 times increase in the sample kept in dark. The enhancement in SOD activity was more in the tissues kept in light than their corresponding dark samples. The catalase (CAT) activity showed an irregular trend in this study (Fig. 2B). Under light at 10 mg/L, the activity significantly (*P*=0.05) increased in comparison to the control, whereas at the same concentration in dark sample, the activity slightly declined in comparison to the dark control sample. Then at 50 mg/L, CAT activity declined significantly both under light and dark and subsequently towards higher concentrations, there was no further change. But the important change noted was that from 50 mg/L onwards, the tissues exposed to light have lower CAT activity than the dark samples. The unspecific peroxidase (POX) activity in this study was assayed taking guaiacol as the reduced co-substrate. There was continuous increase in the POX activity up to 100

mg/L of $HgCl_2$ under light and up to 50 mg/L under dark conditions (Fig. 2C). More than 2.5 times increase in POX activity was found in the light samples at 100 mg/L of $HgCl_2$ in comparison to the control and at 200 mg/L, the activity significantly declined. In the dark tissues, there was about 1.6 times increase in POX activity at 10-50 mg/L $HgCl_2$ in comparison to the control and then onward the activity declined. In the control and in the tissues treated with 10 mg/L $HgCl_2$, the dark samples showed more POX activity than the corresponding light one. But at all other higher concentrations, the light samples had more POX activity than the corresponding dark samples and at 100 mg/L $HgCl_2$, the difference in POX activity between light and dark samples was quite prominent.

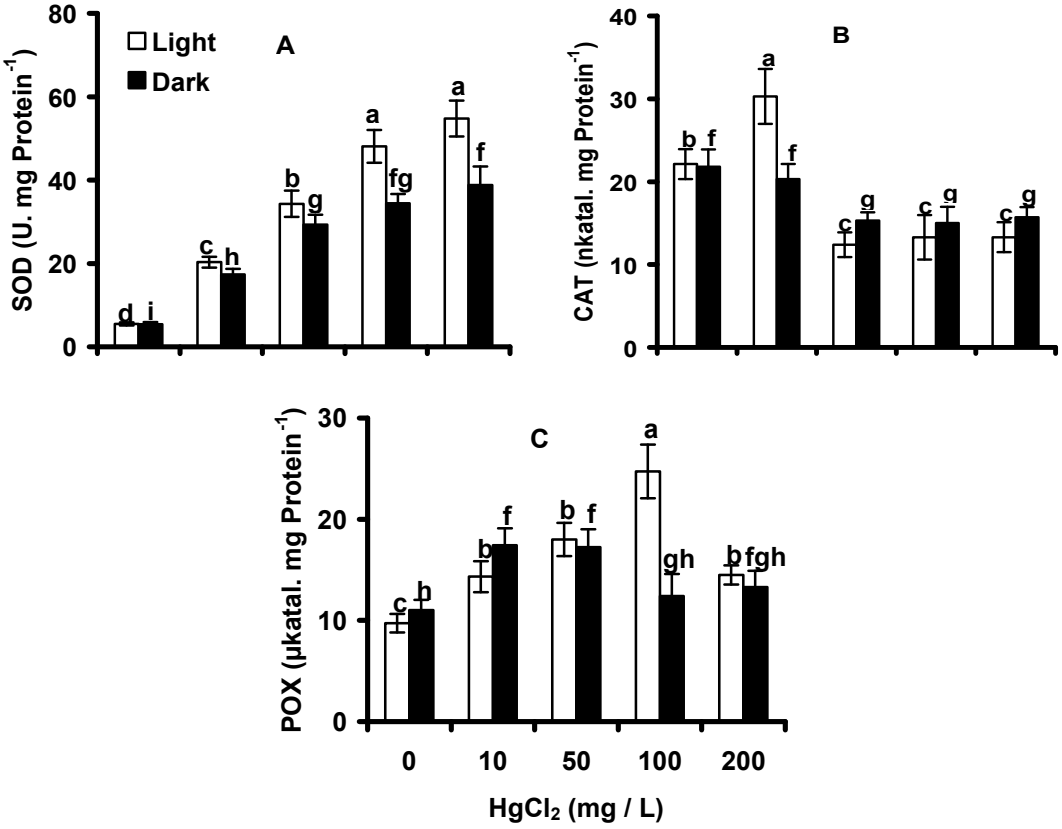

Fig. 2. Changes in the activities of (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) guaiacol peroxidase (POX) in *Hydrilla verticillata* (L.f.) Royle twigs exposed to $HgCl_2$ for 3 h under light and dark conditions.

Rest of the explanations is same as the legend of Fig. 1.

Malondialdehyde (MDA), a decomposition product of peroxidized polyunsaturated fatty acid component of membrane lipid was measured to assess the level of lipid peroxidation, taking thiobarbituric acid (TBA) as the reactive material. The results presented in Fig. 3 revealed that with increase in the metal concentrations, there was increase in lipid peroxidation levels up to 100 mg/L and then there was decline, both under light and dark conditions. The increase in lipid peroxidation reached significant level even at 10 mg/L of $HgCl_2$. It was also noted that at all concentrations tested, the tissues exposed to light have more lipid peroxidation level than the corresponding dark samples.

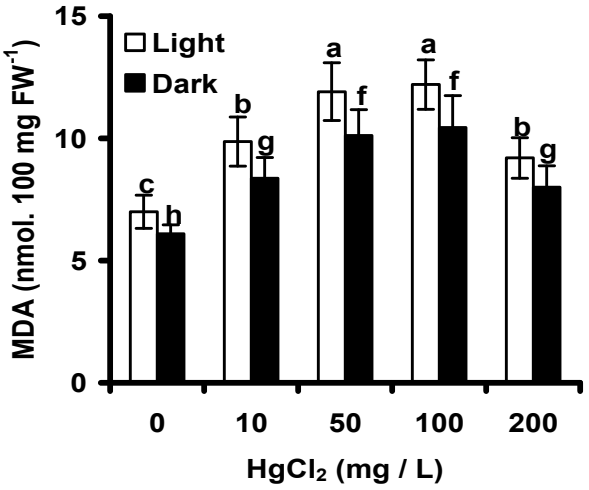

Fig. 3. Changes in the lipid peroxidation level in *Hydrilla verticillata* (L.f.) Royle twigs exposed to $HgCl_2$ for 3 h under light and dark conditions.

Rest of the explanations is same as the legend of Fig. 1.

4. DISCUSSION

Like other abiotic stresses, heavy metal stress has also drawn much attention of the plant scientists since it affects the normal physiology of the plants in many ways. Because of their water solubility, most of the heavy metals are absorbed and accumulated by plants, even though they are not required for their normal metabolic processes. When their concentrations exceed certain tolerance limit, physiological anomalies are observed and under acute cases they may also become fatal. Mercury is one of the most toxic heavy metal and today, the major sources of it for the general environment includes the incineration of wastes and the burning of coals for the production of electricity [2]. Through the effluents of the industries like paper and pulp, chloralkali, plastic, pesticide etc. mercury mainly enters directly into the water bodies or through seepage and thus pollute them. Since this metal is non-essential for the normal metabolic process, studies on the physiological response of plants and particularly that of the submerged aquatic one has attracted the attention of plant physiologists.

In this study, the loss of total chlorophyll has been found in the twigs with increase in mercury concentration in the medium (Fig. 1A) and at 200 mg/L of $HgCl_2$ the loss was visually noticeable in the form of bleaching. At all the concentrations, the loss was more significant in samples kept in light than their respective dark samples. The loss of chlorophyll in *H. verticillata* has also been reported in other studies under exposure to Hg [18], Cu [19] and Ni [20]. The loss of chlorophyll due to exposure to other heavy metal like Cr in wheat has also been reported in other study [21]. According to Shaw [22], loss of chlorophyll is an indication of the prevalence of oxidative stress situation in plants. Therefore, in this study imposition of oxidative stress due to Hg toxicity could be presumed.

Oxidative stress situation is generally a regulated process where the equilibrium between the generation of reactive oxygen species (ROS) and their subsequent scavenging by endogenous antioxidative protective system determines the fate of the organism. The different ROS includes superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical

(·OH) and singlet oxygen ($^1O_2$) which are generated during normal oxygen metabolism. When their concentrations increase significantly, as occur under different developmental stages and also under stress situations, beyond the capacity of cell's endogenous antioxidative protective system to scavenge them off, an oxidative stress situation is imposed in the system [7]. The antioxidative enzymes like SOD, CAT and POX play important role in protecting the cell against oxidative damage. The SOD dismutates superoxide radical to H_2O_2 and O_2. The continuous increase in SOD activity, as reported herein, indicated that the protection against $O_2^{\cdot-}$ was maintained in the tissues in response to mercury stress (Fig. 2A). Similar increase in SOD activity has also been reported by several workers under Cd [23], Al [24], Pb [9], Cu [19,25] toxicity. The increased SOD activity under Hg stress might be due to *de novo* synthesis of SOD protein in the tissues. The increased SOD activity gives more protection against superoxide radicals, but at the same time higher amount of H_2O_2 is also generated in the tissues as dismutation product. Therefore, protection against H_2O_2 is very vital.

Catalase (CAT) decomposes H_2O_2 to H_2O and O_2 in catalatic mode or it can also use up H_2O_2 to oxidize substrates like methanol, ethanol, formaldehyde, formate etc. in peroxidatic mode. It is very difficult to saturate CAT with H_2O_2 due to its high Vmax for destruction of H_2O_2. The higher concentrations of H_2O_2 favours catalatic mode, whereas the lower concentrations favours peroxidatic mode of CAT action. This heme containing enzyme of the aerobic cell plays an important role in the decomposition of H_2O_2 generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, glyoxylate cycle and purine catabolism [7]. The increased CAT activity at 10 mg/L light sample (Fig. 2B) might be due to the induction in the synthesis of the enzyme where the system might have tried to adjust with the Hg imposed stress situation. Increased CAT activity has also been reported in *Hydrilla* under exposure to Cu (up to 1.0 µM concentration) till 7 d [19]. But the decrease in CAT activity towards higher concentrations of Hg (Fig. 2B), and also as have been observed in different systems exposed to metals [26,19,9] might be due to various reasons. These include inactivation of the enzyme protein due to ROS generation [27], decreased enzyme synthesis or alteration in the enzyme subunits' assembling [28]. In a situation with enhance SOD activity, higher CAT activity is highly essential to prevent the H_2O_2 build up in the cell. But the reduced CAT activity found in this study due to $HgCl_2$ concentrations of 50 mg/L onwards favoured the accumulation of H_2O_2 in the tissues. H_2O_2 is known to involve in the transduction of stress signals in cyanobacteria in oxidative stress situations [29]. More CAT activity at 10 mg/L $HgCl_2$ and less activity from 50 mg/L onwards in light samples in comparison to their respective dark counterparts, as observed in this study, indicate that light has an additional effect in altering the CAT activity under Hg stress. Recently Michelet, et al. [30] have reported that high light causes decrease in CAT activity in *Chlamydomonas reinhardtii* that is linked to the reduction state of the photosynthetic electron transport chain allows increase in the H_2O_2 concentration. This accumulated H_2O_2 induces a signaling event that is transmitted to the nucleus and modulates the expression of protection enzymes.

Peroxidases are antioxidative enzymes that also help in the removal of H_2O_2 by the co-oxidation of reduced co-substrates in a reaction mechanism analogous to peroxidatic mode of catalase action. The activities of these enzymes have been frequently used as metabolic indicators in a wide range of physiological studies involving biotic and abiotic stresses. The significant increase in POX activity with increase in the Hg concentrations in the medium (Fig. 2C) should have endowed with some protection against H_2O_2 in the tissues. But there are different reasons behind augmentation in POX activities in plants under stress situations. This may be due to increased release of peroxidases localized in cell walls and such increased peroxidase activities have been reported in rice and wheat under Pb stress [26,9],

in *Hydrilla verticillata* under Cu (up to 1 µM) stress [19], in the shoot tissues of wheat under Cd and Cr stress [9,21]. However, at higher concentration (i.e., at 200 mg/L), there was significant decrease in the POX activity which might be due to the enzyme inhibition and similar decrease in POX activity has also been reported in *Hydrilla verticillata* under exposure to higher concentrations of Cu [19]. Since the increase in POX activity is now used as a biomarker of heavy metal stress [25], the imposition of stress due to Hg in this study could also be presumed.

Ascorbic acid is an important low molecular weight antioxidant of the cell which is known to scavenge the ROS like superoxide radical, hydroxyl radical and singlet oxygen [31]. Decrease in the ascorbic acid content in the tissues due to Hg, as reported herein (Fig. 1C), suggested the decrease in the efficacy of the system to scavenge the reactive oxygen species under Hg stress.

In this study, we found that due to Hg stress, there was some protection against superoxide radical, but protection against H_2O_2 was poor. Hydroxyl radicals are known to generate from H_2O_2 in presence of transition metal ions [7]. Among the different ROS, hydroxyl radical is the most potentially toxic species in the aerobic cells. Among the different cell components, the unsaturated fatty acids of the membrane lipids are highly susceptible to hydroxyl radical attack and are peroxidized. As a result lipid peroxides and hydro peroxides are released causing membrane dissembling, loss of cellular architecture and ultimately cell death. The lipid peroxidation, therefore is considered as a good indicator of prevalence of oxidative stress situation [32]. Even though peroxide level has not been measured in this study, the reported increase in MDA level in the tissues (Fig. 3) indicated that Hg stress increased lipid peroxidation in *Hydrilla verticillata*. However at 200 mg/L $HgCl_2$, there was significant decline in the lipid peroxidation level which might probably be due to limitation in the peroxidizable fatty acid content in the tissues. Like other parameters, the level of lipid peroxidation has also been found to be higher in light samples than their corresponding dark counterparts which suggested that light have some additional effect in increasing Hg toxicity. Thus, the above results suggest the probability of imposition of oxidative stress in the tissues due to Hg toxicity.

In the case of imposed oxidative stress, addition of antioxidant along with stressor (i.e., Hg in this case) is expected to reduce the toxic effect. In this study along with 50 mg/L $HgCl_2$, two different concentrations (i.e., 1 and 10 mM) of one antioxidant (ascorbate) were taken and were found that chlorophyll loss due to Hg was neutralized by ascorbate (Table 1). The addition of 10 mM ascorbate along with 50 mg/L of $HgCl_2$ was found to give much protection to pigment loss (Table 1) and the total chlorophyll content of this sample was almost nearer to control tissue. Ascorbate is known to scavenge superoxide radical, hydroxyl radical and singlet oxygen [31] and serves as an important low molecular antioxidant in the cell. The restoration of total chlorophyll in *Hydrilla* twigs by ascorbate indirectly suggests that there was imposition of oxidative stress in the tissues due to Hg.

5. CONCLUSION

Summing up, the alterations in the levels of different physiological parameters including the activities of antioxidative enzymes, as reported herein, suggest that there was imposition of oxidative stress in the *Hydrilla* tissues by Hg which might be one of the probable mechanisms behind Hg induced toxicity. However, measurement of the activities of enzymes involved in ascorbate-glutathione pathway and the level of other low molecular weight antioxidants like glutathione, α-tocopherol etc. is highly essential to supplement this

proposition. The altered values of the different parameters among the respective light and dark samples further indicated the additional toxic effects of Hg under light. *Hydrilla verticillata* plants have been reported to show high level of tolerance to Hg where they grew well in medium containing up to 5.0 µM Hg for 168 h [18]. In that study the suitability of this plant in monitoring Hg pollution was suggested on account of its uptake potential, tolerance and common occurrence in Hg polluted water bodies. Of course, in comparison to that study, the concentrations of $HgCl_2$ tested in our study is higher, i.e., from 10 mg/L (36.8 µM) on wards, but such a situation can also not be overruled in the environment where there would be continuous influx of wastes containing Hg to the aquatic bodies. Since only 3 h of exposure to Hg, the plant responded severely, exposure to a longer duration would have catastrophic consequence. However, application of antioxidant (e.g., ascorbate) along with Hg has added significant relief to the toxic effects. Therefore, increased antioxidative efficiency of *Hydrilla verticillata* can improve the tolerance of it to higher Hg concentrations. With better uptake potential, this plant can be a suitable candidate for Hg bioaccumulation from polluted water bodies.

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COMPETING INTERESTS

Authors declare that there are no competing interests.

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