Response of antioxidative enzymes to arsenic-induced phytotoxicity in leaves of a medicinal daisy, *Wedelia chinensis* Merrill

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Abstract

Background: *Wedelia chinensis* Merrill (Asteraceae) is a medicinally important herb, grown abundantly in soils contaminated with heavy metals, including toxic metalloid arsenic (As). The leaves have immense significance in treatment of various ailments. **Objective:** The present study was undertaken to ascertain whether the edible/usable parts experience oxidative stress in the form of membrane damage during As exposure or not. **Materials and Methods:** Responses of seven antioxidant enzymes were studied in leaves under 20 mg/L of As treatment in pot experiment. **Results:** When compared to control, activities of superoxide dismutase, monodehydroascorbatereductase, dehydroascorbatereductase, glutathione reductase, and gluathione peroxidase had increased, while the catalase level reduced and ascorbate peroxidase activity changed non-significantly in As-treated seedlings. This suggested overall positive response of antioxidant enzymes to As-induced oxidative stress. Although hydrogen peroxide content increased, level of lipid peroxidation and magnitude of membrane damage was quite normal, leading to normal growth (dry weight of shoot) of plant under Astreatment. **Conclusion:** *W.chinensis* is tolerant of As-toxicity, and thus, can be grown in As-contaminated zones.

Keywords: Antioxidant enzymes, arsenic, medicinal plant, oxidative stress, *Wedelia chinensis*

INTRODUCTION

India is rich in diversity of medicinal plants,[1,2] and daisies (family Compositae or Asteraceae) with their peculiar fruit structure form an integral part of this diverse natural medicine.[3,4] *Wedelia chinensis* Merrill (syn*W.calendulacea* Less) belonging to tribe Heliantheae in the family Asteraceae is widely distributed in India, China, Japan and many south-east Asian countries. It is a procumbent, perennial herb with a bright yellow colored capitulum. The leaves are extensively used for hair tonic, and are purported to be useful in the treatment of cough, cephalagia and in alopecia (skin diseases). A decoction of the herb is also used in uterine hemorrhage and menorrhagia. It is widely used as a cholagogue, and a deobstruent in hepatic enlargement for jaundice and other ailments of the liver and gall bladder.[1,5] Compounds from *W.chinensis* synergistically suppress androgen activity and growth in prostate cancer cells, a major cause of cancer-related deaths in males in Western countries.[6] It is useful in the treatment of osteoporosis of knee and also possesses anti-inflammatory activity. In several rural areas, extract of dries leaves of the plant is applied to wounds as they have wound healing property.[7] The fresh juice from the leaves of *W.chinesis* has been used by Ayurvedic physicians in India for external use to treat skin problems, dermatitis, eczema and acne. The annual demand of botanical raw drugs in India has been estimated at 319,500 MTfor the year 2005-06, and it is steadily increasing.[8]

Arsenic (As) is ubiquitous in many environments and highly toxic to all forms of life. In recent times, the impact of irrigation with high As groundwater on soil and crop has drawn more attention due to transfer of As to the food chain via groundwater-plant-soil system.[9,10] The bioaccumulation of As in different crops including legumes, cereals and herbal medicinal plants has a huge negative impact for public health issues in both rural and urban population,[11-13] and this is of great environmental concern because As is known to be a carcinogen and a powerful co-mutagen.[14,15]

When a plant experiences environmental stress, the critical balance between the formation of reactive oxygen species (ROS) and the quenching activity of different antioxidants is disturbed. Despite being a non-redox-active metalloid, As exposure induces the generation of ROS in plants[16-18] through its intraconversion from one ionic form to be other.[19] The excess ROS generated by As-induced stress unbalance the cellular redox system in favor of oxidized forms, leading to oxidative damage to membrane lipids.[17,20] To combat oxidative stress, plants modulate a series of enzymatic and non-enzymatic antioxidant defense mechanisms.[21-24] Superoxide dismutase (SOD) constitutes the first line of defense by dismutating the superoxide radicals, a major ROS, but produces hydrogen peroxide (H_2O_2) as an end product. The H_2O_2 is another ROS, and it is generally removed by the action of ascorbate perodixase (APX) and catalases. In the ascorbate-glutathione cycle, monodehydroascorbate reductase (MDAR) and dehydroascorbate reductase (DHAR) reduce ascorbate, while glutathione reductase (GR) plays a crucial role by maintaining the reduced glutathione (GSH)/ oxidized glutathione (GSSH) ratio in favor of ascorbate reduction.[24] Gluathione peroxidase (GPX) scavenges lipid peroxides preferably using thioredoxins over GSH as an electron donor.[24]

In order to counter the detrimental impacts of As, two strategies have been considered: Removal of As through efficient strategies such as phytoremediation and the development of safe crops that can be grown in contaminated soils.[17] Both of these scenarios would require understanding of the mechanistic details of perception of As-induced stress and ensuing tolerance.[25] Members of Asteraceae family exhibited the capacity to detoxify heavy metals and salt stress,[26-28] but by contrast with other metals, the biochemical responses of this group of plants to As are not well understood. Low amount of As have been traced in leaves and shoots of *W.chinensis* Merrill plants,[29] but the antioxidant defense response of As-induced phytoxicity is not known in this medicinal herb. Leaf being the most essential component for preparation of different herbal drugs from *Wedelia*, is used for the biochemical analysis in the present investigation. The main objectives of this study were to assess the extent of oxidative damage on membrane lipid and to elucidate the response of seven antioxidative enzymes, namely SOD, APX, MDAR, DHAR, GR, CAT and GPX in leaves of *W.chinensis* seedlings under a selected dose (20 mg/L) of As.

MATERIALS AND METHODS

Plant material and treatment

Fresh shoot samples with roots in nodes were collected from various open fields at Chakdaha (23°5'0" N, 88°31'0"E), Nadia, West Bengal, India. The specimen was authenticated (Prof. Sobhan Kr Mukherjee, Department of Botany, University of Kalyani, Kalyani) and a voucher specimen (VL/RP/Bot/11) was preserved at the departmental herbarium of R.P.M. College, Uttarpara, West Bengal, India for future reference. After proper washing with tap water the plant samples were directly transferred to earthen pots (30 cm in diameter) containing field soils and leaf mould mixture at 1:3 ratios and watered evenly for three consecutive days. Initially, pots were kept away from direct sunlight, and watered very carefully to keep the soil moist but not wet. After proper establishment of the plant (one plant/pot) the pots were kept in a randomized block design in a growth chamber with controlled environment (14-h photoperiod, 28/18(±2°C), relative humidity of 70 ± 2% and a photon flux density of 200 μmol/m^2 s). After 7 days of transplantation in pots, seedlings were subjected to As treatment. Pots were watered (3.1 L in each pot) every alternate day with arsenate contaminated water as a solution of $Na_2HAsO_4.7H_2O$ (Sigma-Aldrich, Bangalore, India) in deionized water at a concentration of 0 mg As/L (control treatment) and 20 mg As/L. The treatment dose was selected after a preliminary experiment in similar condition. Plants were harvested 20 d after imposition of treatment, and oven dried at 72°C for 48 h. Dry weight of shoot was measured. The complete experiment was repeated 3 times under the same set of conditions with three replications for each treatment. Fresh, fully opened leaves were used for analysis of different biochemical parameters.

Analysis of lipid peroxidation

Lipid peroxidation rates were determined by measuring the malondialdehyde (MDA) equivalents following the method of Hodges *et al*.[30] About 0.5 g of fresh leaf tissue was homogenized in a mortar with 80% ethanol. The homogenate was centrifuged at 3000 ×*g* for 12 min at 4°C. The pellet was extracted twice with the same solvent. The supernatants were pooled and 1 ml of this sample was added to a test tube with an equal volume of either the solution comprised of 20% trichloro acetic acid (TCA) and

0.01% butylatedhydroxy toluene (BHT) or solution of 20% TCA, 0.01% BHT and 0.65% TBA. Samples were heated at 95°C for 25 min and cooled to room temperature. Absorbance was measured at 532 nm. Lipid peroxidation rate was expressed as nmolMDA g/FW.

Hydrogen peroxide estimation

H_2O_2 content of leaves was measured following the methodology as described previously.[31] Tissue samples were homogenized in the extraction medium 0.1 M K-phosphate (pH 6.4) supplemented with 5mM KCN. The assay mixture contained 250 μM ferrous ammonium sulphate, 100 μM sorbitol, 100 μM xylenol orange, and 1% ethanol in 25 mM H_2SO_4. Changes in absorbance were determined by the difference in absorbance between 550 nm and 800 nm, and H_2O_2 contents were calculated from a standard curve.

Electrolyte leakage

Electrolyte leakage (EL) was assayed by measuring the ions leaching from leaf tissue into deionized water.[32] Fresh leaf samples (100 mg) were cut into small pieces (about 5 mm segments) and placed in test tubes containing 10 ml deionized water. Tubes were kept in a water bath at 32°C for 2 h. After incubation, electrical conductivity (EC_1) of the bathing solution was recorded with an electrical conductivity meter (Systronics M-308, Kolkata, India). The samples were then autoclaved at 121°C for 20 min to completely kill the tissues and release all electrolytes. Samples were then cooled to 25°C and final electrical conductivity (EC_2) was determined. The EL was expressed as EL (%) = $(EC_1/EC_2) \times 100$.

Antioxidant enzyme assays

Fresh leaf tissue (1 g) was homogenized in 3 mL of 50 mM potassium phosphate buffer, pH 7.8, containing 1 mM Ethyelene diamine tetraacetate, 1 mMdithiothreitol and 2% (w/v) PVP with chilled mortar and pestle in an ice bath, as described earlier.[33] The homogenate was centrifuged in a refrigerated centrifuge at 15,000 × *g* for 20 min. The resultant supernatant was used as source of enzyme. The extraction was performed at 4°C. For measuring ascorbate peroxidase activity, the leaf tissue was separately ground in homogenizing medium containing 2.0 mMascorbate in addition to the other ingredients.

SOD (EC 1.15.1.1) activity was determined by the nitro-blue tetrazolium (NBT) photochemical assay method as described by Beyer and Fridovich.[34] The reaction mixture (3 mL) contains 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 μM NBT, 0.1 mM EDTA, 2 μM riboflavin and 0.1 mL of enzyme extract. The absorbance of the solution was measured at 560 nm in a UV-Vis spectrophotometer. SOD activity was expressed as Enzyme unit/milligram protein. One unit of SOD was defined as the amount of protein causing a 50% NBT photoreduction.

APX (EC1.11.1.11) activity was assayed following the method of Nakano and Asada.[35] The reaction mixture (3 mL) contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mMascorbate, 0.1 mM H_2O_2 and 0.1 mL enzyme extracts The H_2O_2-dependent oxidation of ascorbate (AsA) was followed by a decrease in the absorbance at 290 nm with extinction constant 2.8/Mmcm. APX activity was expressed as nmolAsA oxidized/minutes milligram protein.

Activity of MDAR (MDAR, EC 1.6.5.4) was assayed according to Miyake and Asada.[36] Monodehydroascorbate was generated by the ascorbate oxidase in a reaction mixture of 1 mL containing 50 mM Hepes-KOH buffer (pH 7.6), 0.1 mM NADPH, 2.5 m Mascorbate, ascorbate oxidase (0.14 U) and suitable aliquots of enzyme extract. MDAR activity was expressed as μmol NADPH oxidized/minutes milligram protein.

GR (GR, EC 1.6.4.2) activity was measured according to the method of Carlberg and Mannervik[37] by monitoring the glutathione-dependent oxidation of NADPH. The reaction mixture contained 0.75 mL 0.2M potassium phosphate buffer (pH 7.0, with 2 mM EDTA), 75 μL 2.0 mM NADPH and 75 μL GSSH (20 mM) in a cuvette. Reactions were initiated by adding 0.1 mL enzyme extracts to the cuvette, and the decrease in absorbance at 340 nm was recorded for 2 min. GR activity was expressed as μmol NADPH oxidized/minutes milligram proteinwith the extinction coefficients for NADPH of 6.2/mMcm.

DHAR (DHAR, EC 1.8.5.1) activity was measured following Nakano and Asada.[35] The complete reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 2.5 mM GSH, 0.2 Mm dehydroascorbate (DHA) and 0.1 mM EDTA in a final volume of 1 ML. Increases in absorbance was recorded at 30 s interval for 3 min at 265 nm. Enzyme activity was expressed as μmolAsA formed/minutes milligram protein.

CAT (EC 1.11.1.6) was extracted in 50 mM K-phosphate buffer (pH 7.0) and 0.5% PVP-10, and its activity was assayed by measuring the reduction of H_2O_2 at 240 nm (ε=39.4/Mcm) for 1 min.[38]

Total GPX (EC 1.15.1.1) activity was determined from 1 g plant tissues extracted in 3 mL of 0.1 M Tris-HCl, pH 7.5, containing 2 mM DTT and 1 mM EDTA. The enzyme activity was ascertained by using cumenehydroperoxide (both selenium and non-selenium enzyme types) as a substrate and GR coupled assay to monitor the oxidation of

GSH.[39] The assay mixture consisted of 0.1 M phosphate buffer, pH 7.0, containing 0.2% (w/v) Triton X-100, 0.24 unit of GR, 1 mM GSH, 0.15 mM NADPH, and 1 mM cumenehydroperoxide. After addition of enzyme eluate, cuvettes were incubated at 30°C for 10 min and NADPH was added to measure the basal rate of GSH oxidation by monitoring the absorbance at 340 nm for 3 min. The reaction was initiated by addition of cumenehydroperoxide, and GPX activity was expressed as change in absorbance at 340 nm/minutes milligram protein.

Statistical analysis

The results are presented as mean values ± standard errors in three replications. Statistical significance between mean values was measured by simple "*t*-test", using the Microsoft Excel "data analysis 2007" tool pack. A probability of $P < 0.05$ was considered significant.

RESULTS

Effect of arsenic on plant growth, lipid peroxidation, H_2O_2content and membrane leakage

Plant growth as measured by shoot dry weight was quite normal in As-treated seedlings [Table 1]. Change in lipid peroxidation, as measured by malondialdehyde (MDA) contents, and percentage of electrolyte leakage (EL) was not significant ($P < 0.05$) in seedlings of As-treated *Wedelia* plants, compared to control level [Table 1]. However, nearly 1.4-fold increase was recorded in tissue H_2O_2 content in the plants under As treatment [Table 1].

Antioxidant enzyme activity

Alterations in activities of seven antioxidant enzymes in control and 20 mg/L As treatments are presented in Table 1. Under stress condition, leaf SOD activity increased significantly (1.7-fold) in the treated plants, while activity of the H_2O_2-decomposing enzyme APX remained unchanged. Compared with control plants, activity of MDAR, GR and DHAR enzymes increased significantly in treated plants. MDAR and GR exhibited an approximately 1.8-fold and 1.6-fold increase in their activity, respectively, while more than the 2-fold increase was measured for DHAR under As-treatment. CAT activity, however, declined by 2-fold, while GPX level increased 5-fold over control value under As treatment [Table 1].

Table 1: Effect of arsenic treatments (0 mg/land 20 mg As/L) on shoot dry weight, lipid peroxidation, hydrogen peroxide, electrolyte leakage and antioxidant enzyme activities in *Wedelia chinensis* Merrill

Parameters+	0 mg/L (control)	20 mg As/L
Shoot dry weight (g/plant)	5.2±0.09	4.9±0.11
Lipid peroxidation (nmolmalondialdehyde/g FW)	1.9±0.01	2.03±0.06
Hydrogen peroxide content (μmol/g DW)	5.22±0.10	7.38±0.17*
Electrolyte leakage (%)	12.50±0.78	15.77±1.03
SOD (U/mg protein)	210.3±9.8	357.5±10.0*
APX (nmolAsA oxidized min/mg protein)	190.6±7.5	193.3±5.9
MDAR (μmol NADPH oxidized/min mg protein)	0.29±5.6	0.51±4.0*
GR (μmol NADPH oxidized/min mgprotein)	0.10±0.08	0.16±0.11
DHAR (μmolAsA formed/minmg protein)	0.76±1.6	1.61±2.8*
CAT (nmol/min mg protein)	39.9±11.7	20.6±10.9*
GPX (nmol NADPH/minmg protein)	7.8±3.5	41.7±5.0*

+Data are means±standard error of three replicates, *Significant at *P*<0.05 level, SOD: Superoxide dismutase, APX: Ascorbateperodixase, MDAR: Monodehydroascorbate reductase, GR: Glutathionereductase, DHAR: Dehydroascorbatereductase, CAT: Catalases, NADPH: Reduced nicotinamide adenine dinucleotide phosphate, GPX: Gluathioneperoxidase, AsA: ascorbate

DISCUSSION

Cell membrane stability is often related to As tolerance in plants, and the conductance measurement of EL from leaf cells is usually used as an indicator of membrane damage in As-treated plants.[40] Change in cell membrane integrity is closely linked with extensive membrane lipid peroxidation in plants.[41-43] There is significant evidence that exposure to inorganic As species results in the generation of ROS in plants[20,44] through the conversion of arsenate to highly toxic arsenite within plants.[45] ROS, namely superoxide radicals induce the degradation of phospholipids and the resulting polyunsaturated fatty acids released by such a breakdown are then peroxidised.[46] In the present investigation, marginal increase in tissue MDA content under As exposure indicated non-significant change in rate of lipid peroxidation. Presumably, this helped the plant to maintain membrane integrity as evidenced from the percentage of EL in the treated plant as per control level. However, higher level of membrane lipid peroxidation and subsequent leakage due to heavy metal-induced generation of ROS was reported in leaves of several common medicinal plants.[12,47] Metal loading of herbal plant parts is of great concern as it can alter the potency of medicinal plants.[47,48]

Regulation of cellular redox state is a crucial factor when a plant experiences As-induced oxidative stress. The production of different classes of ROS including H_2O_2 under different environmental stresses triggers the synthesis of enzymatic antioxidants within the plant cells.[42,43,45] In the present study, increased SOD activity helped the *Wedelia* plant to quench superoxide radicals under As treatment.However, as has frequently been pointed out, that activity of SOD converts one ROS to

another ROS (H_2O_2). H_2O_2 is a predominant oxidant within cell, and its over-accumulation cannot be allowed in an environment where thiol-regulated enzymes are functioning.[31,33,43] Increased SOD activity coupled with the unchanged level of APX activity and low CAT level might be responsible for higher accumulation of H_2O_2 in leaves of As-treated *Wedelia* plants than control plants. Although a positive correlation was usually found between H_2O_2 accumulation and lipid peroxidation as an obvious indication of oxidative stress,[31] no such relationship was evidenced in the present study. Quite remarkably, MDA content and percentage of EL in As-treated *Wedelia* plants were as per control level. Certainly, significant increase in GPX activity in response to As treatment might be instrumental in efficient scavenging of lipid peroxide products, protecting the cell from ROS-induced membrane damage. The complementary role of GPX to low APX activity was reported in an ascorbate-deficient mutant of the legume, grass pea.[33]

Increased MDAR and DHAR activities helped the *Wedelia* seedlings to combat As-induced phytotoxicity, as both the enzymes are responsible for ascorbate reduction in the ascorbate-glutathione cycle. Concomitantly, significant increase in GR activity suggested ability of *Wedelia* seedlings to regenerate GSH and to keep the GSH redox in favor of reducing environment for efficient functioning of antioxidant enzymes under As treatment.

In conclusion, the ability of As-treated *W.chinensis* seedlings to maintain normal growth (measured as dry weight of shoot) in the present investigation clearly demonstrates tolerance of this medicinal plant to high arsenate concentration in soil. The outcome is important in view of the increasing cultivation and trade of medicinal plants and growing contamination of plant parts by heavy metals.[49] As leaves of *W.chinensis*are often used as juice, raw and in paste formation to treat different ailments, its tolerant of high As level assumes significance in this respect.

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- If any of the bibliographic elements are missing, incorrect or extra (such as issue number), it will be shown as INCORRECT and link to possible articles in PubMed will be given.