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RESEARCH ARTICLE

# Analysis of arsenic induced physiological and biochemical responses in a medicinal plant, *Withania somnifera*

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**Abstract** *Withania somnifera* has been an important herb in the Ayurvedic and indigenous medical systems for centuries in India. However, these grow as weeds mostly in the wastelands, which receive contaminated water from municipal and industrial sources. In the present investigation, plants of *Withania somnifera* were exposed to various concentrations of arsenate (AsV) and arsenite (AsIII) (0, 10, 25, 50, 100 μM) for 10 days and analysed for accumulation of arsenic (As) and physiological and biochemical changes. Plants showed more As accumulation upon exposure to AsIII (320 μg $g^{-1}$ DW in roots and 161 μg $g^{-1}$ DW in leaves) than to AsV (173 μg $g^{-1}$ DW in roots and 100 μg $g^{-1}$ DW in leaves) after 10 days of treatment. Consequently, AsIII exposure caused more toxicity to plants as compared to that AsV, as evaluated in terms of the level of photosynthetic pigments and oxidative stress parameters (superoxide, hydrogen peroxide and lipid peroxidation), particularly at higher concentrations and on longer durations. Plants could tolerate low concentrations (variable for AsIII and AsV) until longer durations (10 days) and high concentrations for shorter durations (1–5 days) through increase in antioxidant enzymes and by augmented synthesis of thiols. In conclusion, As tolerance potential of *Withania* plants on one hand advocates its prospective use for remediation under proper supervision and on the other demonstrates possible threat of As entry into humans due to medicinal uses.

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## Introduction

Arsenic (As) is a ubiquitously distributed and an extremely toxic metalloid that has come into limelight as a consequence of demonstration of its accumulation in groundwater and in a number of edible (rice in particular) and medicinal plants (Chakraborti et al. 2002; Tripathi et al. 2007; Saper et al. 2008; Dwivedi et al. 2010; Srivastava et al. 2014). Arsenic bioavailability and toxicity to plants are strongly dependent on its chemical form. In general, inorganic As species viz., arsenate (AsV) and arsenite (AsIII) are considered to be more toxic than organic ones because of their chemical analogy to essential ions like phosphate and borate (Zhao et al. 2009). Arsenate is taken up by plants through high-affinity phosphate transporters and disrupts energy flows in cells. The uptake of AsIII occurs through aquaglyceroporins and in the cytosol, AsIII reacts with sulfhydryl groups of enzymes and proteins (Finnegan and Chen 2012). Due to chemical analogy with essential elements, plants cannot avoid its entry; nonetheless plants can regulate As levels at and after entry to certain extent and can also combat As toxicity. Both As species are also known to induce the production of reactive oxygen species (ROS) beyond control levels, which are combated by plants by stimulation of various enzymes and compounds that function as antioxidants (Srivastava et al. 2007; Trease and Evans 1989). In addition, plants complex As in the form of AsIII through S-containing ligands like glutathione (GSH) and phytochelatins (PCs) and transport the AsIII-thiol complexes into vacuoles to reduce the free AsIII concentration (Srivastava et al. 2007). Arsenic toxicity starts to appear at an As level when both thiol and

antioxidant systems fail to counteract As load and this specific As level varies from plant to plant from one As species to other (Srivastava et al. 2012).

Lately, the therapeutic use of herbal medicines is gaining considerable momentum in the world. Herbal medicines act as the major remedy in traditional system of medicine and have been used in medical practices since antiquity. India is one of the largest producers of traditional medicines in the international market but most of the medicinal plants are grown without much care (Boderkar 2004). Medicinal herbs sometimes grow as weeds in the contaminated wastelands and many of the medicinal plants have been found to accumulate significant amount of As whether collected from field (Tripathi et al. 2012) or grown in lab conditions (Chaiyarat et al. 2011). In our earlier study (Siddiqui et al. 2013), we demonstrated significant As accumulation in three species of *Ocimum* (*O. tenuiflorum*, *O. basilicum* and *O. Gratissimum*). Another important medicinal plant is *Withania somnifera* Dunal (Family: Solanaceae), which has an abundance of secondary metabolites (Thakur et al. 1989). It is popularly known as Ashwagandha and its roots are important source of steroidal alkaloids and steroidal lactones (Qamar Uddin et al. 2012). It grows profusely in India and other parts of the world, is used traditionally for anti-tumor activity, hepatoprotectiveness and anti-inflammatory activity (Christina et al. 2004). The present study was designed to evaluate the As accumulation potential of *Withania somnifera* and to study ensuing physiological and biochemical changes of the plants.

## Material and methods

### Plant material and treatment conditions

Seeds of *Withania somnifera* (L.) Dunal were obtained from CSIR-Central Institute of Medicinal and Aromatic Plants, Lucknow (CSIR-CIMAP), India. Seeds were grown in plastic pots (12 in. diameter) filled with acid washed sand (0.01 M HCl), and were placed in glass house receiving normal day light, temperature and humidity. These pots were irrigated daily with 10 % Hoagland's solution. After 30 days of sowing, plants having approximately same height and weight were carefully uprooted and washed with tap water and then in double distilled water. Then, plants were transferred into 250 ml conical flasks containing 10 % Hoagland's solution and allowed to grow for one week in laboratory conditions (light:dark, 14:10 h, temperature 28±2 °C, with 115 μmol $m^{-2}$ $s^{-1}$ illumination provided through fluorescent tube lights). Experiments were set up in triplicate and each replicate contained five plants of equal size. Plants were treated with different concentrations of AsV (0, 10, 25, 50, 100 μM; $Na_2HAsO_4$) and AsIII (0, 10, 25, 50, 100 μM; $NaAsO_2$) for a period of 10 day. At harvesting time points (1, 5, and 10 day), plants were washed with double distilled water, blotted to remove water, and roots and leaves (4th leaves from the top were used uniformly) were separated, which were used for the study of various parameters.

### Quantification of arsenic

For analyzing the level of absorbed As, plants were initially washed with ice-cold deionized water to remove the adsorbed As followed by drying to constant weight at 80 °C for 2 days in a hot air oven. Total As in the plant material was estimated after digestion of oven-dried plants (100 mg) following the protocol detailed in Srivastava and D'Souza (2010). The As concentrations in the solutions used for treatments were also analyzed after preparation (i.e., prior to exposure to plants) and the measured concentrations of As were found to be in range of 95–98 % of nominal concentrations.

### Estimation of photosynthetic pigments

Photosynthetic pigments were extracted in 80 % chilled acetone as per the procedure of Arnon (1949). Carotenoid concentration in these extracts was calculated by the formula given by Duxbury and Yentsch (1956).

### Estimation of water status

Water status was evaluated in the leaf tissue of plants by measuring Relative water content (RWC). For the determination of RWC, fresh weight, saturated weight and dry weights were measured as described by Slavik (1974). The RWC was calculated by following formula:

$$\mathrm{RWC} = \frac{\text{Fresh weight} - \text{Dry weight}}{\text{Saturated weight} - \text{Dry weight}} \times 100$$

### Estimation of proline

Proline content was estimated by following the method of Bates et al. (1973) in supernatant using acid-ninhydrin reagent. Plant material (500 mg) was homogenized in 5 ml 5′-sulphosalicylic acid (3 %) and centrifuged at 10,000×g for 10 min at 4 °C. Reaction mixture containing 2 ml supernatant, 2 ml ninhydrin reagent and 2 ml acetic acid (glacial) was heated at 100 °C for 1 h. The reaction was terminated on an ice bath and reaction mixture was extracted with toluene with vigorous mixing. The absorbance was recorded at 520 nm. Proline content was calculated from the standard curve prepared using known concentrations of proline (L-proline, sigma) and is expressed as μmol $g^{-1}$ fresh weight.

### Measurement of metalloid induced oxidative stress

The oxidative stress experienced by the plants was measured in terms of level of reactive oxygen species [Superoxide radicals ($O_2^{\circ-}$) and hydrogen peroxide ($H_2O_2$)] and lipid peroxidation [malondialdehyde (MDA)]. The rate of $O_2^{\circ-}$ production, the level of $H_2O_2$ and MDA were measured following the protocols of Chaitanya and Naithani (1994), Pick (1986), and Heath and Packer (1968), respectively as described earlier (Mishra et al. 2008; Srivastava et al. 2011).

### Activities of antioxidant enzymes

Plant material (500 mg) was homogenized in 100 mM potassium phosphate buffer (pH 7.0) containing 0.1 mM EDTA and 1 % polyvinylpyrrolidone (*w*/*v*) at 4 °C. Homogenate was centrifuged at 15,000×g for 15 min at 4 °C. Supernatant was used to measure the activities of enzymes. Protein content of the supernatant was measured by the protocol of Lowry et al. (1951). The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), guaiacol peroxidase (GPX) and catalase (CAT) were assayed by following the protocols of Beauchamp and Fridovich (1971), Nakano and Asada (1981), Smith et al. (1988), Hemeda and Klein (1990), and Aebi (1974), respectively as described in detail earlier (Srivastava et al. 2006).

### Thiolic compounds

Among thiolic compounds, total non-protein thiols (NP-SH), cysteine and total glutathione (reduced: GSH and oxidized: GSSG) levels were measured. The analysis of NP-SH content was performed following the protocol of Ellman (1959). For the analysis of cysteine, plant material (500 mg) was homogenized in 5 % chilled perchloric acid and centrifuged at 10,000×g for 10 min at 4 °C. Cysteine content was measured in supernatant using acid–ninhydrin reagent according to the method of Gaitonde (1967). For GSH and GSSG determination, plant material (500 mg) was frozen in liquid nitrogen homogenized in 0.1 M sodium phosphate buffer (pH 8.0) containing 25 % meta-phosphoric acid. The homogenate was centrifuged at 20,000×g for 20 min at 4 °C and GSH content was determined fluorometrically in the supernatant (Hissin and Hilf 1976).

### Statistical analyses

Experiments were performed in a complete randomized block design involving five treatments and two durations. A two-way analysis of variance was performed to confirm the validity of the data by using Microsoft Excel version 2007.

## Results and discussion

### Arsenic accumulation and effects on plant growth, photosynthetic pigments, and water status

The accumulation of As was correlated to both concentration and duration of the treatment. However, it varied depending on the species of As. Total As accumulation was higher in plants exposed to AsIII than to AsV and roots accumulated more As in comparison to leaves. The maximum accumulation of As after 10 day at 100 μM in roots was 320 μg $g^{-1}$ DW and 173 μg $g^{-1}$ DW in plants exposed to AsIII and AsV, respectively. The corresponding values in leaves were 161 μg $g^{-1}$ DW and 100 μg $g^{-1}$ DW, respectively (Fig. 1a and b). As a result of higher accumulation of As, plants exposed to AsIII experienced greater toxicity (Fig. 2b) than plants exposed to AsV (Fig. 2a). The present results of differential As accumulation in AsIII and AsV exposure are in concurrence with earlier studies (Srivastava et al. 2007) and are supported by the fact that AsIII and AsV show variable accumulation under similar conditions due to differences in the mode of uptake and transport (Abedin et al. 2002; Zhao et al. 2010). Previously, the potential of *Withania* plants to accumulate Cu (Khatun et al. 2008), Cr, Zn, Mn, Ni, and Pb (Khan et al. 2008) has been demonstrated. Other medicinal plants like *Ocimum* have also been found to accumulate significant amount of metals including As (Angelova et al. 2007; Siddiqui et al. 2013). As pointed by in our earlier study on *Ocimum* (Siddiqui et al. 2013), the significant As tolerance potential and consequent As accumulation in high amounts by *Withania somnifera* warrants towards possible risks associated with uncontrolled and unregulated use of herbs for medicinal purposes. In a recent work, Kulhari et al. (2013) analyzed heavy metal levels in commonly used medicinal plants from differential locations in India. However, they found that most of the naturally growing herbs accumulated metals within permissible limits except for Cr in some samples. Kulhari et al. (2013) did suggest that medicinal plants used for medicinal purposes should not be procured from contaminated areas. Similarly, Khan et al. (2007), who conducted heavy metal accumulation analysis in *W. somnifera* plants collected from contaminated fields, proposed to perform proper regulatory checks before their use for medicinal purposes. It becomes more so important in the light of a recent study showing protective effect of *W. somnifera* against As induced deleterious effects in rats proposing use of ashwagandha for medical uses. Hence, *W. somnifera* possesses wide range medical applications and the only precautionary step which must be taken is its proper check before use.

Toxic effects of AsIII and AsV were evaluated in terms of photosynthetic pigments and water status. Both AsIII and AsV negatively affected the levels of total chlorophylls and carotenoids in a concentration and duration dependent manner

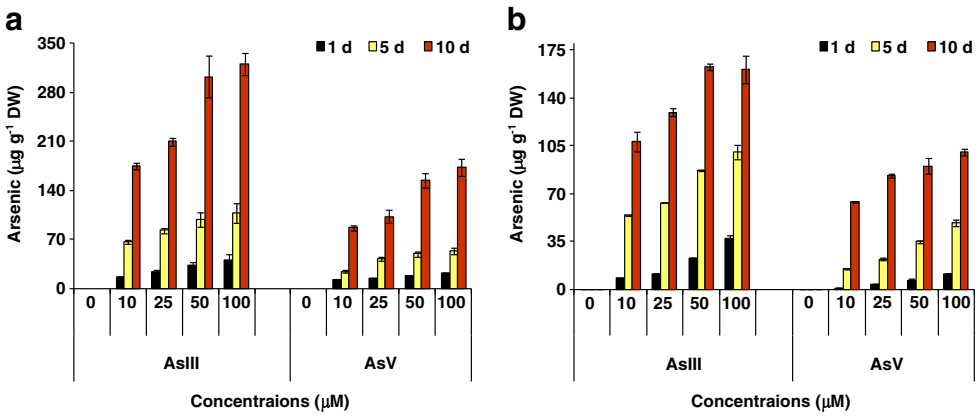


**Fig. 1** Arsenic accumulation in roots (**a**) and leaves (**b**) of *W. somnifera* after 1, 5 and 10 day of exposure to different concentrations of arsenite and arsenate. Values represent the mean of three biological replicates±S.D. Two-way ANOVA was significant at $p \leq 0.01$

and AsIII caused a greater damage as compared to AsV (Fig. 3a and b). At the maximum exposure concentration of 100 μM, the decline in total chlorophylls and carotenoids showed an increasing trend of 7 to 83 % and 13 to 71 % respectively upon AsIII exposure, and 1 to 60 % and 4 to 47 %, respectively upon AsV exposure (Fig. 3a and b). Arsenic is known to alter pigment concentrations through negative effects on an important biosynthetic enzyme like δ-aminolevullinic acid dehydratase (Jain and Gadre 2004) and also by inducing the activity of degrading enzyme; chlorophyllase (Jain and Gadre 1997). The disturbed status of nutrients upon As exposure has also been proposed as the reason for decrease in pigment levels (Li et al. 2006). The present results agree with the earlier reports showing decline in photosynthetic pigments as a consequence of As exposure (Srivastava et al. 2007, 2013a; Mishra et al. 2014). The reduction is photosynthetic pigments could further lead to reduced growth (Mishra et al. 2014), which would affect the water status or vice versa (Srivastava et al. 2013b). Similar to pigments, water status, measured as RWC was also affected to a greater extent upon AsIII exposure (22 %) than upon AsV exposure (16 %) at 100 μM at 10 day (Fig. 3c). The observed decrease in RWC could be ascribed to effect on the functioning of aquaporins of plasmamembrane intrinsic protein (PIP) class, which are major channels for water uptake, as reported earlier by Srivastava et al. (2013b).

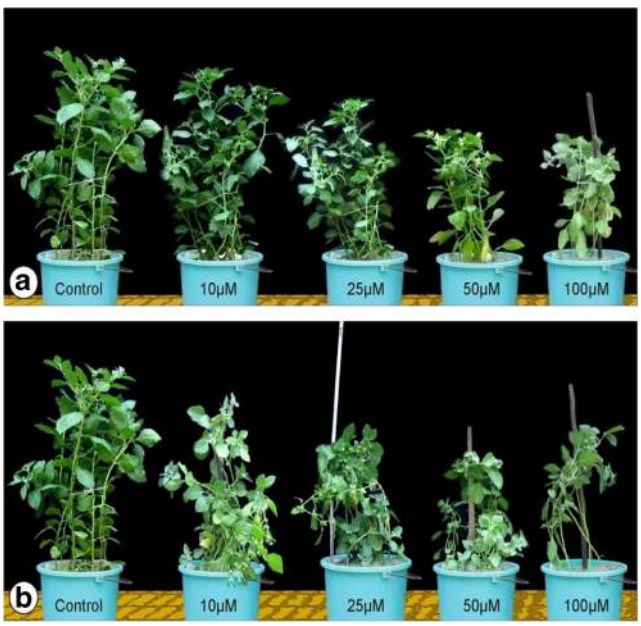


**Fig. 2** Effect of different concentrations of arsenate (**a**) and arsenite (**b**) on growth pattern of plants after 10 day of exposure

## Effect of arsenic on oxidative stress parameters and response of antioxidant enzymes

In order to further assess the toxic effect of the metalloid species, changes in the level of superoxide anion, hydrogen peroxide ($H_2O_2$) and malondialdehyde (MDA: as a measure of lipid peroxidation of membranes) were monitored. The level of superoxide anion, $H_2O_2$ and MDA showed an increase in the concentration and duration dependent manner upon exposure to both AsIII and AsV; however superoxide anion demonstrated slight decline in response to AsV until 5 days. The maximum increases in superoxide anion, $H_2O_2$ and MDA were observed at the maximum concentration (100 μM) and the longest duration (10 day), which were 90, 99 and 165 %, respectively in response to AsIII and 51, 66 and 76 %, respectively in response to AsV. Hence, both As species caused significant induction of production of reactive oxygen species (ROS) and consequent damages on membrane stability through peroxidation of membrane lipids. This might be attributed to channeling of majority of glutathione (GSH) for towards As detoxification and its shortage for antioxidant functions (Mylona et al. 1998), disturbed photosynthetic efficiency of plants (Mishra et al. 2014), outpacing of ROS production with ROS removal with increasing As concentration (Srivastava et al. 2007), disturbance of the balance of adenine and pyridine nucleotides and altered energetic and redox equilibria (Srivastava et al. 2011; 2013c) or increase in the activity of pro-oxidant enzymes like NADPH oxidase, ascorbate oxidase, lipoxygenase (Srivastava and D'Souza

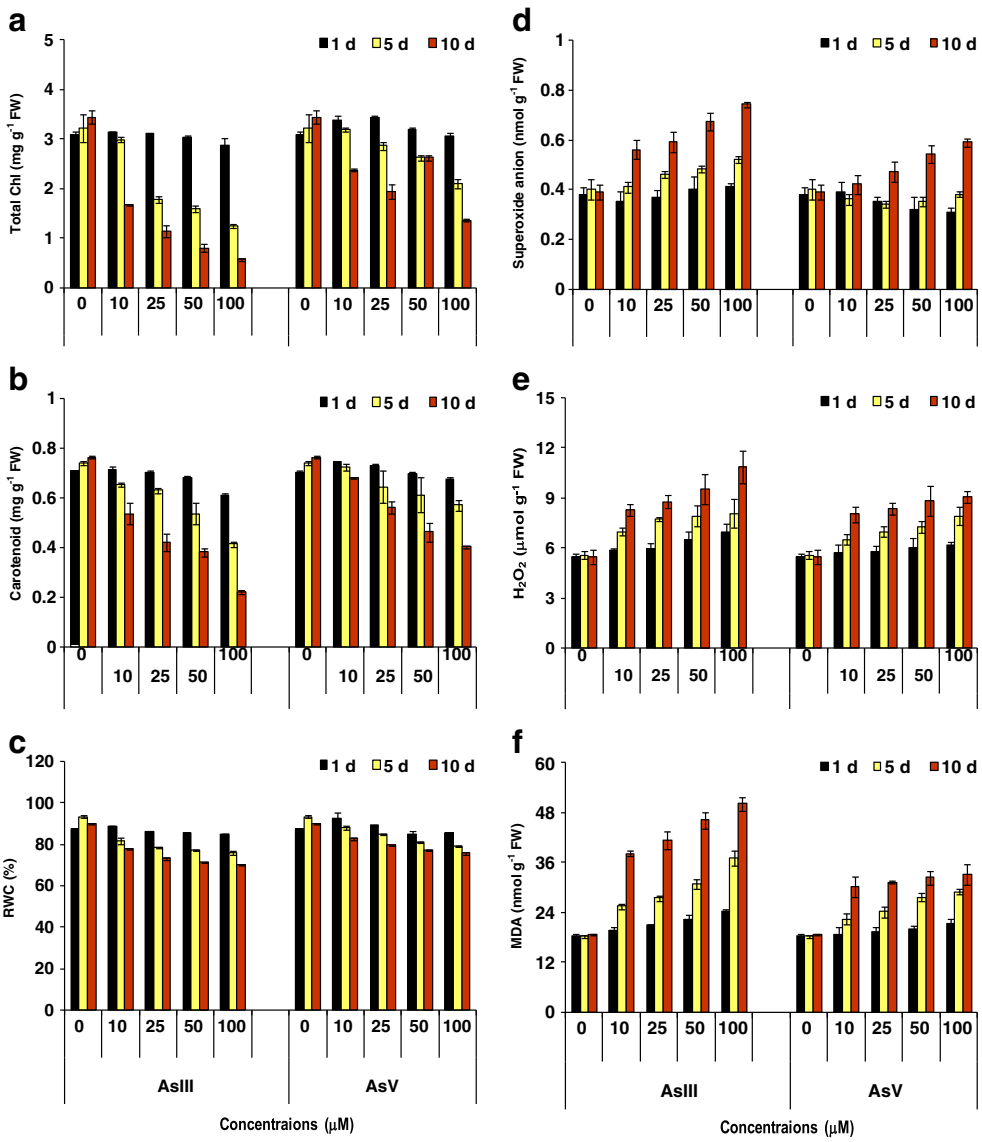


**Fig. 3** Effect of different concentrations of arsenite and arsenate on total chlorophyll (**a**) and carotenoid (**b**) levels and RWC (**c**), and superoxide anion (**d**), hydrogen peroxide (**e**) and MDA (**f**) levels in leaves of *W. somnifera* after 1, 5 and 10 day of exposure. Values represent the mean of three biological replicates±S.D. Two-way ANOVA was significant at $p \leq 0.01$

2010; Srivastava et al. 2011) and glycolate oxidase (Gupta et al. 2013). The variable response of ROS and MDA levels was attributable to differences in total amount of As accumulation and the rate of As uptake under AsIII and AsV exposure; both of which affect plant's potential for As tolerance (Hartley-Whitaker et al. 2001; Srivastava et al. 2007). In earlier study on *W. somnifera* studying Cu responses also, a continuous increase in superoxide anion, $H_2O_2$ and MDA levels were noticed (Khatun et al. 2008).

To assess the response of plants to oxidative stress, various antioxidant enzymes and proline levels were analyzed (Fig. 4). The studied enzymes and proline showed significant increases at different concentrations and durations in response to both AsV and AsIII; however the increases in antioxidants were more pronounced in plants exposed to AsV than to AsIII. The SOD activity demonstrated significant increase only up to 50 µM at 1 day and up to 25 µM at 5 days in response to AsIII while it declined progressively at 10 day (Fig. 4a). APX activity showed declining trend in most of the treatments in response to AsIII except at 10 µM (Fig. 4b). GR activity was increased on all concentrations at 1 day and up to 25 µM at 5 days but declined beyond 10 µM at 10 day (Fig. 4c). In contrast, AsV-exposed plants demonstrated significant increase in the activities of SOD, APX and GR on all concentrations and durations except at 100 µM at 10 day (Fig. 4a, b and c). GPX activity did not demonstrate a positive response upon exposure to both AsV and AsIII and showed decline in most of the treatments except significant increase noticed at a single point (25 µM AsV at 5 days) (Fig. 4d). CAT activity also demonstrated a differential response. In response to AsIII, significant increase was noticed only at 10 µM at 5 days while AsV exposure induced an increase on all treatments except at 100 µM at 10 day (Fig. 4e). Proline levels however increased in response to both AsIII and AsV on all concentrations and

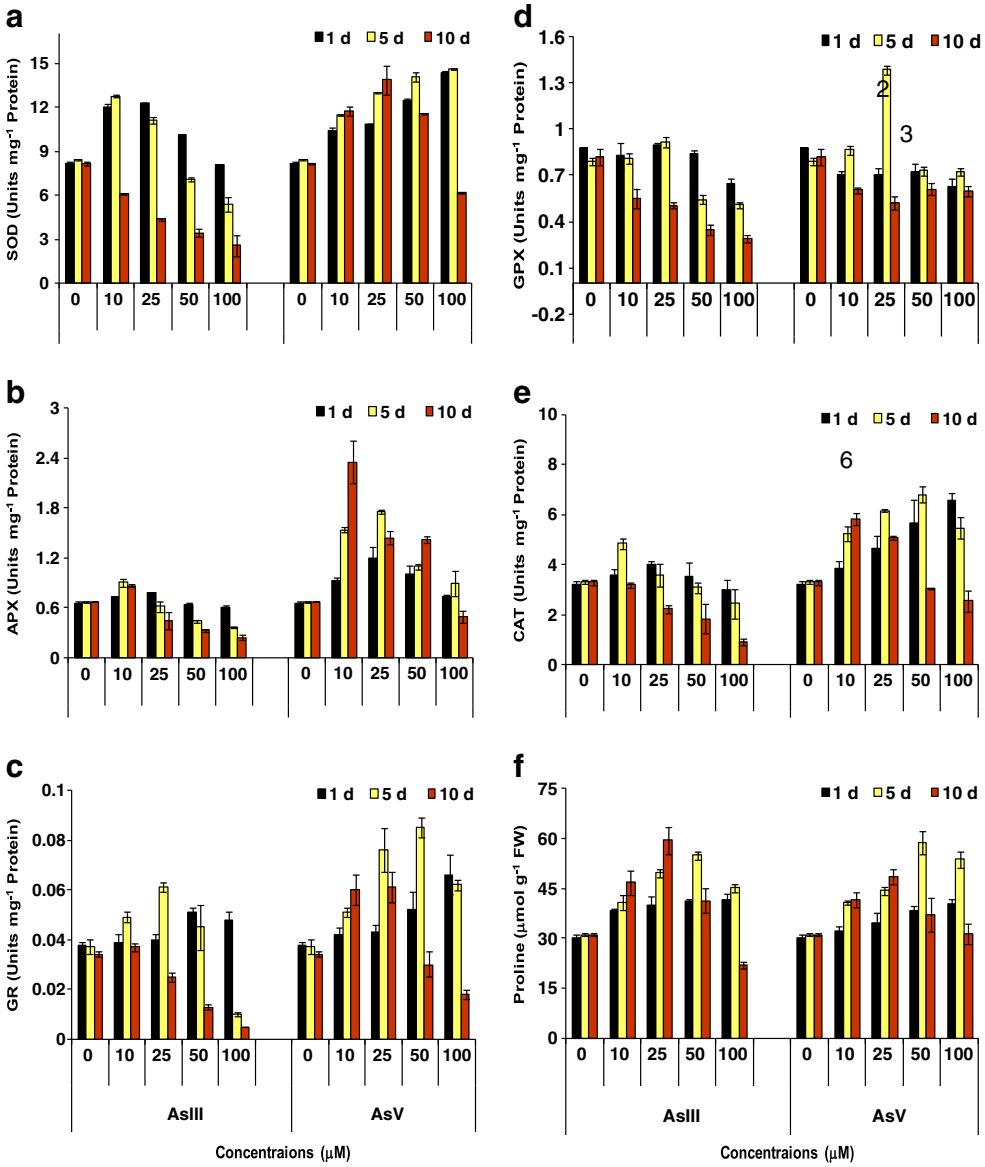


**Fig. 4** Effect of different concentrations of arsenite and arsenate on the activity of SOD (**a**), APX (**b**), GR (**c**), GPX (**d**) and CAT (**e**) and on the level of proline (**f**) in leaves of *W. somnifera* after 1, 5 and 10 day of exposure. Values represent the mean of three biological replicates±S.D. Two-way ANOVA was significant at $p \leq 0.01$

duration except at 100 µM AsIII at 10 day (Fig. 4f). Studying the response of antioxidant enzymes in *Hydrilla verticillata*, Srivastava et al. (2007) too noticed higher increases in the activities of antioxidant enzymes upon exposure to AsV compared to AsIII. An important pathway involved in combating ROS production at cellular and organellar (chloroplast and mitochondria) level in plants is the ascorbate-glutathione pathway and APX and GR are the parts of this pathway (Foyer and Noctor 2011). A proper coordination among the activities of SOD, APX and GR has been found to lead to regulated ROS levels (Srivastava et al. 2007). In this study, concerted increases in SOD, APX and GR were observed upon AsV exposure but the same was not true in case of AsIII. Hence, dismutation of superoxide anion and H2O2 was apparently taken care of in a better way in plants exposed to AsV than to AsIII. Two other important enzymes for controlling levels of $H_2O_2$ and lipid peroxides are CAT and GPX; both of which did show more response upon exposure to AsV than to AsIII. Hence, antioxidant system could not be properly stimulated to achieve the desired regulation over increasing ROS and this possibly contributed to greater oxidative stress observed in plants exposed to AsIII than to AsV. Higher activities of various enzymes in AsV exposed plants at lower doses might be responsible for the observed better growth of plants in response to AsV compared to AsIII. The proposed hypothesis of lower rate of AsV uptake than AsIII giving more time and hence allowing plants to handle the As stress in a better way (Hartley-Whitaker et al. 2001; Srivastava et al. 2007) holds true to the present study also. The present results are also in concurrence to the earlier study of Khatun et al. (2008) where Cu stress was found to induce various antioxidant enzymes at lower concentrations followed by a decline at higher

concentrations in W. *sominfera*. Proline is an important amino acid in plants, which has multiple functions in plants with the most important being the role as an osmoticum under water stress conditions and hence protecting cytoplasmic enzymes and stabilizing membranes (Verbruggen and Hermans 2008). However, it is also known today for its participation in scavenging ROS and in assisting plants combat oxidative stress (Matysik et al. 2002). An increase in proline level has been noticed upon As exposure in earlier works on *H. verticillata* (Srivastava and D'Souza 2010), *Spinacea oleracea* (Pavlik et al. 2010), *Vigna mungo* (Srivastava and Sharma 2013), and rice (Kumar et al. 2014). AsIII and AsV induced almost similar increases in proline levels and hence with respect to the response of proline, no differential pattern was found.

## Effect of arsenic on thiol compounds

The level of NP-SH increased significantly upon exposure to both As species with increase occurring up to 25 μM followed by decline. However, in plants exposed to AsV, NP-SH level did not decline to lower than control level at any treatment while AsIII exposure did induce decline in NP-SH to close to control level or lower than that at higher exposure concentrations and durations. Further, at equimolar concentrations, plants exposed to AsV showed more increase as compared to AsIII (Fig. 5a). The cysteine content, however, did not demonstrate much significant response upon exposure to either AsIII or AsV. The maximum increase in cysteine level was 11 % by AsV at 100 μM and 5.8 % by AsIII at 25 μM, at 5 days. At 50 μM and 100 μM, AsIII exposure led to significant decline in cysteine level (Fig. 5b). Similar to NP-SH, comparatively better response of GSH was observed in response to AsV than AsIII considering the extent of increase at equimolar concentrations of AsIII and AsV (Fig. 5c). GSSG levels increased at all concentrations of both the species and on all durations in comparison to control; the extent of increase being more in plants exposed to AsIII than to AsV (Fig. 5d). As a consequence, GSH/GSSG ratio, an important indicator of redox balance of cells, was found to decline significantly beyond 25 μM at 1 day and beyond 10 μM at 5 and 10 days in response to AsIII. In contrast, in plants exposed to AsV, only 50 and 100 μM caused a significant decline in GSH/GSSG ratio beyond 1 day (data not shown). Thiol metabolism plays important role in combating As stress (Mishra et al. 2008) as thiols including GSH not only complex As through sulfur (Mishra et al. 2013) and reduce the concentration of free As and allow its transport to vacuoles (Song et al. 2010) but they also assist in the maintenance of redox status of the cell (Foyer and Noctor 2011), which is crucial for the functioning of various metabolic reactions (Srivastava et al. 2011). Cysteine is the primary metabolite of sulfur assimilation pathway, which is then consumed for the synthesis of GSH and subsequently, GSH molecules are polymerized to produce phytochelatins (PCs) (Srivastava et al. 2012). The

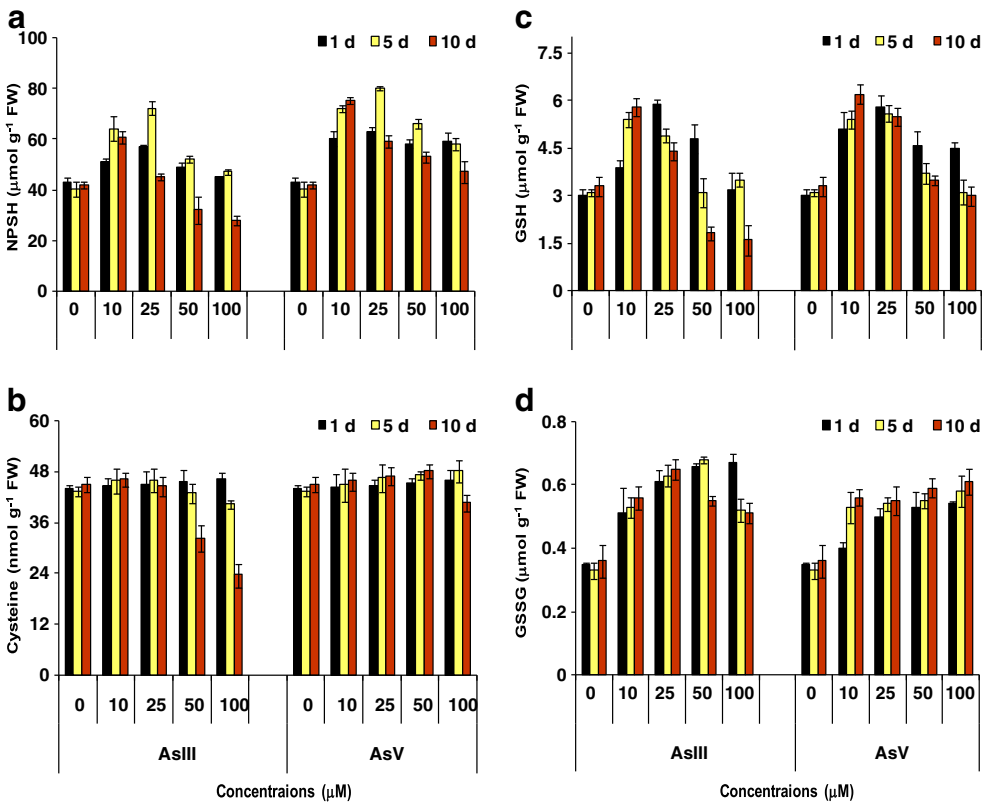


**Fig. 5** Effect of different concentrations of arsenite and arsenate on the level of NP-SH (**a**), cysteine (**b**), GSH (**c**) and GSSG (**d**) in leaves of *W. somnifera* after 1, 5 and 10 day of exposure. Values represent the mean of three biological replicates±S.D. Two-way ANOVA was significant at $p \leq 0.01$

present results indicated that AsV exposure not only induced thiols to a higher level but also maintained GSH/GSSG ratio through the stimulation of GR activity (Fig. 4c) as compared to that of AsIII. Hence, in plants exposed to AsV, As complexation by thiols would have been more efficient as compared to that of AsIII. This, in addition to higher influx of As, appeared to have resulted in observed greater toxicity to plants in response to AsIII as compared to AsV.

## Conclusion

In conclusion, *W. somnifera* plants tolerated concentration of AsV and AsIII that were higher than normally present in contaminated areas and plants accumulated high amount of As in roots followed by leaves. Therefore, *W. somnifera* can be grown in As polluted soil for remediation purposes under strict regulation. However, the use of As containing plants in medicinal preparation is not advised due to its toxic effect and health risk. Further, our results are laboratory based and before exploiting the results in field, a pilot field study is recommended.

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**Conflict of interest** Authors declare that they have no conflict of interest.

**Authors contribution** F. Siddiqui conducted all experiments and analyzed data. P.K. Tandon conceptualized and planned the study. F. Siddiqui and S. Srivastava contributed in preparing the final manuscript.

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