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GROWTH AND METABOLIC RESPONSES OF *GLYCINE MAX* L. TO ARSENATE AND ARSENITE: A COMPARATIVE ASSESSMENT

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Abstract

Arsenic (As), a non-essential metalloid and severely toxic to all the living organisms exists mainly in two inorganic forms arsenate (As^{V}) and arsenite (As^{III}). Arsenic is known to cause deleterious impacts on growth and metabolism of plants chiefly *via* slowing down the cell division and elongation, increased formation of reactive oxygen species (ROS) and alteration in antioxidative system. Therefore, the present study was aimed to evaluate the adverse effects of both As^{V} and As^{III} on growth traits, contents of As, ROS and malondialdehyde, and antioxidant system in *Glycine max* L. Data revealed that As reduced germination percentage, radicle length and biomass accumulation, while enhanced the contents of As, malondialdehyde, and localization and accumulations of ROS. In addition, significant change in the activities of antioxidant enzymes and proline content were revealed. Overall results suggested that As^{III} is more injurious to *G. max* L. than As^{V}.

Introduction

Arsenic (As) has been considered as an element of environmental concern in the recent past, due to its toxicity and carcinogenic properties. A huge part of agricultural land is contaminated with As, where its concentration ranges from 3.34 to 105 mg/kg soil (Patel *et al.* 2005). Both bioavailability and toxicity of As are dependent closely on its chemical form. Two inorganic forms arsenite (As^{III}) and arsenate (As^{V}) are severely toxic than the organic ones, and in between these inorganic forms, former being more injurious than the later (Chandrakar *et al.* 2017a). In general, As^{V} is taken up by the root cells through phosphate transporters. It replaces phosphate during ATP synthesis thereby disturbs the energy flow (Siddiqui *et al.* 2015). While, As^{III} enters into the root cells *via* aquaporins and reacts with -sulphydryl (-SH) groups of both enzymes and proteins, thus altering their functions (Armendariz *et al.* 2016). Hence, As adversely affects normal metabolic processes of the plants such as growth, development, respiration, photosynthesis, reproduction, etc. (Chandrakar *et al.* 2016a). Therefore, in the recent past research on As toxicity has come to limelight to unravel the precise mechanisms of its toxicity on plants and human beings.

Both As^{III} and As^{V} are popularly shown to provoke formation of reactive oxygen species (ROS) like superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (Chandrakar *et al.* 2016b). These ROS can lead to peroxidation of membrane lipids, oxidation of proteins, damage to nucleic acids, inhibition of enzymes, activation of apoptotic pathway, finally terminating with death of the cells or tissues (Rughani *et al.* 2016, Yadu *et al.* 2016, 2017a). To fight against oxidative damages, plant cells are armed with protective mechanisms comprising both enzymatic and non-enzymatic members. Enzymatic system includes superoxide dismutase (SOD: EC 1.15.1.1), catalase (CAT: EC 1.11.1.6), ascorbate peroxidase (APX: EC 1.11.1.11), etc., while glutathione, phytochelatins, proline, etc., constitutes non-enzymatic defense system (Chandra and Keshavkant 2016, Yadu *et al.* 2017b).

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Glycine max L. is an important legume crop grown globally. In India and in many other developing countries, a significant part of the land often used for its cultivation is reported to have higher As concentration than the permissible limit i.e. 20 mg/kg soil (Patel *et al.* 2005). Therefore, present study was designed to monitor changes in growth attributes, As content, oxidative stress markers like $O_2^{\cdot -}$, H_2O_2, $^{\bullet}OH$ and malondialdehyde (MDA), and members of antioxidative system (SOD, CAT, APX and proline) in *G. max*, under As^{III} and As^{V} treatments.

Materials and Methods

Eighty seeds of *Glycine max* L. (variety JS 335) collected locally were disinfected with 0.1% (v/v) sodium hypochlorite solution for 5 min following washing (5 times) with *MilliQ* water (MW) (Millipore, Gradient A-10, USA). These seeds were then placed for germination over two layers of filter paper soaked with MW (control) and two concentrations; 25 and 100 µM, each of As^{III} (sodium arsenite) and As^{V} (sodium arsenate), in germination boxes of 26 × 16 × 3 cm size (Chandrakar *et al.* 2017b). These concentrations were selected taking into account the fact that most of the agricultural fields used for cultivating *G. max* L. contain similar or higher As concentration (Patel *et al.* 2005). These boxes were then kept in darkness at 32°C. After every 24 hrs, seeds were supplied with 10 ml each of respective treatment solutions. On fifth day of treatments, germination percentage was assessed and then radicles were removed from the seeds. The lengths of the ten randomly selected radicles were measured. Likewise, ten radicles were pooled in each replicate and weighed in an electronic balance. Dry mass (DM) of these radicles was measured after placing them in an oven at 60°C for 72 hrs. Each experiment was repeated five times.

To determine As, 0.1 g of dried radicles were digested using HNO_3:H_2O_2:H_2O in the ratio of 5 : 1 : 1 (v : v : v) at 80°C until a transparent solution was obtained (Chandrakar *et al*. 2017a). The volume of digested sample was made up to 15 ml with MW, and amount of As in it was monitored using atomic absorption spectrometer coupled to a hydride generation system (Agilent, AA240, USA). The standard reference material of As (Merck, Darmstadt, Germany) was used for calibration and quality assurance for analysis.

For $O_2^{\cdot -}$ determination, 0.2 g radicles were imbibed in 100 ml of 20 mM sodium phosphate buffer (pH 6), consisting 500 µM XTT, in the dark at 26ºC, on a shaker (100 *g*). Absorbance of the soaking medium was read at 470 nm in a UV-Vis spectrophotometer (Lambda 25, Perkin Elmer, USA) (Schopfer *et al.* 2001). Content of $O_2^{\cdot -}$ was calculated using an extinction coefficient 2.16 × 10^4/mol/cm and expressed as µmol/g fresh mass (FM).

To measure $^{\bullet}OH$, 0.2 g radicles were homogenized with 2 ml of sodium phosphate buffer (10 mM, pH 7.4) comprising 15 mM 2-deoxyribose and incubated at 37ºC for 2 hrs. To this mixture, thiobarbituric acid (TBA, 0.5% (w/v) in 5 mM NaOH) and glacial acetic acid were added (Chandrakar *et al.* 2016a). Next, this complex was heated at 100ºC for 30 min and then cooled completely at 4ºC. Absorbance was recorded at 532 nm and corrected for non-specific absorbance at 600 nm. Content of $^{\bullet}OH$ was calculated using an extinction coefficient of 0.155/M/cm and expressed as nmol/g FM.

Weighed amounts (0.2 g) of radicles were extracted with 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged (11 000 *g*, 15 min) (Velikova *et al.* 2000). Supernatant was added to equal volumes of sodium phosphate buffer (10 mM, pH 7) and potassium iodide (1 M), and absorbance was read at 390 nm. Content of H_2O_2 was calculated using extinction coefficient 0.28/µmol/cm and expressed as µmol/g FM.

Sites of $O_2^{\cdot -}$ and H_2O_2 production in *G. max* L. radicles were localized by nitroblue tetrazolium (NBT) and 3,3-diaminobenzidine (DAB) staining, respectively (Rughani *et al.* 2016).

To determine MDA, radicles (0.1 g) were homogenized with 20% (w/v) TCA consisting 0.5% (w/v) TBA. Homogenate was boiled for 30 min at 100ºC, cooled and centrifuged (11000 g, 10 min) (Velikova *et al*. 2000). Absorbance (at 532 nm) of the supernatant was recorded and MDA content was expressed as nmol/g FM.

To extract enzymes, radicles (0.2 g) were homogenized with 10 mM sodium phosphate buffer (pH 7.2) consisting 1 mM EDTA, 2 mM DTT and 0.2% (v/v) Triton X-100, and centrifuged (12000 *g*, 20 min) at 4ºC. Supernatant thus obtained was used for estimation of antioxidants.

Activity of SOD was determined by estimating the per cent inhibition of pyrogallol auto-oxidation by the enzyme at 420 nm (Marklund and Marklund 1974). A unit of SOD activity was defined as the amount of enzyme that inhibited 50% of NBT photoreduction. Its activity was expressed as units of SOD/min/mg protein.

Activity of CAT was assayed by recording the decomposition of H_2O_2 by the enzyme at 240 nm (Chance and Maehly 1955). Activity of CAT was expressed as nmol/min/mg protein.

Activity of APX was determined by recording the rate of ascorbate oxidation at 290 nm (Nakano and Asada 1981). Its activity was referred as mmol/min/mg protein.

Electrophoretic separation of SOD, CAT and APX were performed over native-PAGE (10%) using tris-glycine buffer (5 mM, pH 8.3) (in case of APX, running buffer consisted of 4 mM ascorbate), at 4ºC for 2 hrs with a constant current of 20 mA, using mini-protean tetra cell (BioRad, USA). After run, gels were stained with respective solutions, and then imaged and analyzed using a Gel-Doc (BioRad, USA).

To visualize the SOD, gels were incubated in the dark for 20 min in the 2.45 mM NBT solution, and were then immersed in 36 mM dipotassium hydrogen phosphate (pH 7.8) comprising 28 µM each of riboflavin and TEMED, until the gel turns blue except the region showing SOD activity (Chandrakar *et al.* 2016a). To analyze CAT, gels were incubated in 0.03% (v/v) H_2O_2 for 10 min and then rinsed quickly in MW and stained in a solution containing 1% (w/v) each of potassium ferricyanide and ferric chloride. As soon as a green color began to appear, gels were washed with MW (Woodbury *et al.* 1971). For detection of APX, gels were equilibrated with 50 mM sodium phosphate buffer (pH 7) and 2 mM ascorbate for 30 min (Mittler and Zilinskas 1993). Afterwards, the gels were incubated with 50 mM sodium phosphate buffer (pH 7) containing 4 mM each of ascorbate and H_2O_2, for 20 min. Finally, gels were washed twice with sodium phosphate buffer (50 mM, pH 7) and stained in 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT.

Proline was estimated using L-proline as standard (Bates *et al.* 1973). For this, 0.5 g radicle was homogenized with 10 ml of 3% (w/v) sulfo-salicylic acid and centrifuged (6 000 g, 15 min, 26ºC). Supernatant (2 ml) was mixed with equal volumes of ninhydrin reagent and glacial acetic acid, and incubated at 100°C for 1 hr. To it, 4 ml of toluene was added, and the chromophore containing toluene was aspirated out and then absorbance of it was recorded at 520 nm taking toluene as a blank. Content of proline was expressed as mg/g FM.

The mean value from two separate experiments with five replications and their standard deviations are shown. Data were analyzed for DMRT, at $p < 0.05$. Analysis of variance was performed using SPSS (Ver 16).

Results and Discussion

Plants which are sensitive to As experience oxidative stress upon exposure, exhibited cellular damage and metabolic disfunctions. Exposure of *G. max* L. radicles to 25 µM As^V declined germination percentage, radicle length and DM by 20, 50 and 58%, respectively, compared to

control (Table 1). While, similar concentration of As^{III} reduced these parameters quite largely (56, 61 and 77%, respectively). Considerable reductions in these traits were caused by 100 µM of As^{III} and As^{V}. However, As^{III} was found to be quite inhibitory than the As^{V}. Arsenite being more toxic than As^{V}, binds to -SH groups of the enzymes and proteins, and cause structural damage which results in reduced rate of germination and growth (Chandrakar *et al.* 2016b). The reason behind this growth inhibition might be the utilization of significant amount of energy for the production of stress responsive elements like phytochelatins and antioxidants, in the stressed tissues. In addition, reduced growth under As-stress might also be related with the lower cell wall elasticity and mitotic activity in the root that declined the rate of cell division, expansion, and elongation of the newly formed cells (Armendariz *et al.* 2016, Chandrakar *et al.* 2016a). Moreover, reduced biomass might also be an outcome of enhanced permeability of cellular membranes, tissue loss and lower uptake of nutrients under As-stress (Chandrakar *et al.* 2017a). Dose-dependent fall in seed germination percentage, radicle length and DM has also been observed in *G. max* seedlings under As^{III}/As^{V}-stress by Armendariz *et al*. (2016).

Table 1. Impacts of arsenite (As^{III}) and arsenate (As^{V}) on germination, radicle length, dry mass, arsenic content, malondialdehyde, superoxide, hydrogen peroxide, hydroxyl radical, and proline in *Glycine max* L.

	Control	25 µM As^{III}	100 µM As^{III}	25 µM As^{V}	100 µM As^{V}
Germination (%)	$100^{a} \pm 0$	$43^{c} \pm 1$	$20^{d} \pm 1$	$80^{b} \pm 2$	$26^{d} \pm 5$
Radicle length (mm)	$65^{a} \pm 2$	$25^{c} \pm 1$	$5^{e} \pm 1$	$32^{b} \pm 1$	$9^{d} \pm 2$
Dry mass (g)	$120^{a} \pm 3$	$27^{c} \pm 1.5$	$16^{d} \pm 1.8$	$50^{b} \pm 6$	$20^{d} \pm 4$
Arsenic content (µg/g DM)	-	$24.89^{a} \pm 0.05$	$31.97^{b} \pm 0.06$	$21.35^{d} \pm 0.04$	$26.15^{c} \pm 0.06$
Malondialdehyde (nmol/g FM)	$1.64^{e} \pm 0.1$	$35.55^{c} \pm 0.03$	$45.5^{a} \pm 0.61$	$24.43^{d} \pm 0.16$	$41.57^{b} \pm 1.04$
Superoxide (µmol/g FM)	$1.68^{e} \pm 0.19$	$12.73^{c} \pm 0.48$	$24.2^{a} \pm 0.48$	$7.89^{d} \pm 0.83$	$17.04^{b} \pm 0.63$
Hydroxyl radical (nmol/g FM)	$8.46^{e} \pm 0.07$	$12^{c} \pm 0.06$	$28.71^{a} \pm 0.05$	$10.72^{d} \pm 0.1$	$20.8^{b} \pm 0.26$
Hydrogen peroxide (µmol/g FM)	$0.36^{e} \pm 0.004$	$0.74^{c} \pm 0.003$	$1.24^{a} \pm 0.009$	$0.57^{d} \pm 0.002$	$1.09^{b} \pm 0.008$
Proline (mg/g FM)	$0.31^{e} \pm 0.006$	$1.37^{b} \pm 0.11$	$2.05^{a} \pm 0.19$	$0.84^{d} \pm 0.09$	$1.16^{c} \pm 0.06$

Each datum represents mean ± Sd of five observations. Small letters indicate significant differences between treatments ($p < 0.05$).

Accumulation of As interferes with various metabolic processes of plants, thus adversely affects growth and development (Chandrakar *et al.* 2016a). Amounts of As measured in the radicles treated with 25 and 100 µM As^{V} were 21.35 and 26.15 µg As/g DM, respectively while with 25 and 100 µM As^{III}, its accumulation increased to 24.89 and 31.97 µg As/g DM, respectively (Table 1). Accumulation of As inside the cells might be due to damaged/ altered cell membranes or cell walls (Singh *et al.* 2015, Chandrakar *et al*. 2016b). In this study, As accrual in *G. max* radicle was found to be more in As^{III} treatment than that of As^{V}. This observation is in agreement with the observations made by Chandra *et al.* (2016).

To further probe the deleterious impacts of As in *G. max* radicles, change in the ROS was monitored. Levels of $O_2^{\cdot -}$, $^{\bullet}OH$ and H_2O_2 increased over control by 914, 145 and 202%, respectively after 100 µM of As^V exposure. Whereas, similar concentration of As^{III} raised these by 1340, 239 and 244%, respectively revealing it more injurious than the As^V (Table 1). Additionally, staining of $O_2^{\cdot -}$ and H_2O_2 by NBT and DAB, respectively also supported these findings, exhibiting more accumulation of blue formazan and brownish reaction product in As^{III} subjected radicles, than the As^V treated (Fig. 1A,B). Arsenic-induced alteration in the redox homeostasis, due to inefficient functioning and/or failure of antioxidant system, had been reported as major factor for over production of ROS in stressed tissues (Singh *et al.* 2015). More of ROS was measured in As^{III} exposed radicles, compared to the As^V, which clearly indicates that former is more injurious than the later. The reason shown for higher toxicity of As^{III} was rapid influx and high reactivity of it (Siddiqui *et al.* 2015). Arsenic-induced differential ROS accumulation had also been reported earlier in different plant species (Singh *et al.* 2015, Siddiqui *et al.* 2015, Chandrakar *et al.* 2016b).

Fig. 1. Histochemical detection of superoxide (A) and hydrogen peroxide (B) using nitroblue tetrazolium and 3,3-diaminobenzidine respectively, in *Glycine max* L. radicles exposed to arsenite and arsenate. (Bar: Fig. 1A, B, C = 6.5 cm, 25 mM arsenite = 2.5 cm, 100 mM arsenite = 0.5 cm, 25 mM arsenate = 3.2 cm, and 100 mM arsenate = 0.9 cm).

Lipid peroxidation is one of the free radical mediated chain reactions resulting in membrane deterioration and MDA accumulation under varied abiotic stresses (Xalxo *et al.* 2017, Yadu *et al.* 2017a). This MDA had been reported to alter membrane functions, decrease plant biomass and perturbs protein structure and antioxidant system (Chandrakar *et al.* 2017a). Addition of 25 and 100 µM each of As^V and As^{III} resulted 1389 and 2434% and 2067 and 2674%, respectively, of MDA formation, than the control (Table 1), suggesting As^{III} to be more damaging. This outcome might be the resultant of low reactivity of As^V to cellular lipid fractions, as compared to As^{III}. Similar results were also revealed in As^{III}/As^V treated seedlings of *Lemna minor* L. by Duman *et al.* (2010) and by *Withania somnifera* by Siddiqui *et al.* (2015).

Exposure to As leads to over accumulation of ROS, therefore, it is not surprising that the plants attempt their best to activate defense system to curb damaging molecules (Armendariz *et al.* 2016). Superoxide dismutase, being a first line of defense, removes $O_2^{\cdot -}$ from the cell. Accumulated data showed that there was a concentration based decline (up to 54%) in the SOD activity in both As^{III} and As^V subjected radicles, as compared to control (Fig. 2A). However, its

activity was higher in the As^{III} subjected samples than that of As^{V}-treated, which can possibly be related with the amount of $O_2^{\cdot -}$ produced in it. Similar trend in the activity of SOD was also observed in *L. minor* under similar set of treatments. Moreover, non-denaturing PAGE profile exhibited seven isoforms of SOD, and their intensities were in accordance with those of spectrometric data (Fig. 2A,B).

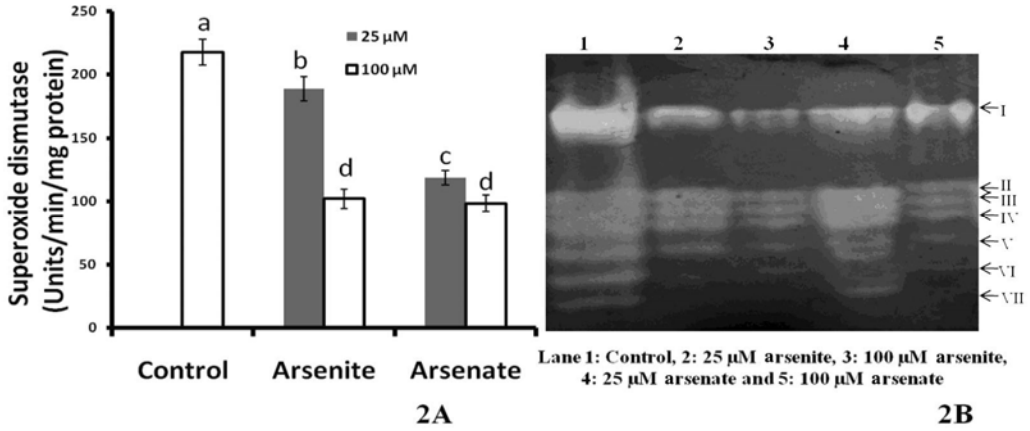

Fig. 2. Effects of arsenite and arsenate on biochemical activity (A) and isoenzymic pattern (B) of superoxide dismutase of the *Glycine max* L. radicle. Each bar represents mean ± Sd of five data. Different letters indicate significant differences between treatments ($p < 0.05$).

In *G. max* radicles, CAT activity declined considerably in parallel to applied concentrations of As. Decline in the activity of CAT was comparatively less in As^{III} subjected radicles than that of As^{V} samples (Fig. 3A, B). Its activity decreased by 41 and 75% in response to 25 and 100 µM As^{III}, respectively, while was 59 and 79% in respect to similar doses of As^{V}, and compared to control (Fig. 3A,B). Similar response had also observed in *Lactuca sativa* L. exposed to different concentrations of As^{III} and As^{V} (Gusman *et al.* 2013). The increased level of H_2O_2 in response to As^{III}, suggested that the higher activity of CAT and/or its isoenzymes may not be sufficient in controlling the excessive amount of ROS. Native-PAGE charting showed two bands of CAT, of which isoform-I followed the trend of biochemical data (Fig. 3A,B).

Activity of APX decreased in response to As^{III}/As^{V} treatments as compared to control. Its total activity did not differ significantly in response to 25 µM of both As^{III} and As^{V}, while was inhibited by 75% under 100 µM of former salt. Srivastava *et al.* (2010) measured higher activity of APX in As^{V}-treated *Brassica juncea* L., than in As^{III} subjected, and was dose dependent also. Native-PAGE profile exhibited four bands of it, displaying varied intensities. Compared to the control, isoform-III was enhanced noticeably in response to As (Fig. 4B).

Proline plays crucial role in the recovery of tissues after their exposure to abiotic stresses (Yadu *et al.* 2016). Findings exhibited significant accrual of proline under As-stress. Of note, this accumulation was more (561%) in the As^{III} supplied *G. max* L. radicles (Table 1). Its maximum (2.05 mg/g FM) was recorded in 100 µM As^{III} supplied samples.

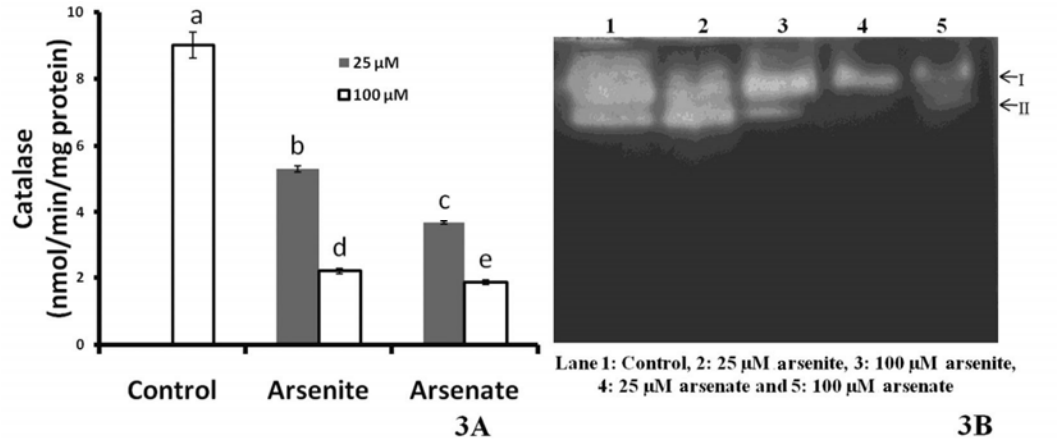

Fig. 3. Changes in the activity (A) and isoenzymes (B) of catalase in the radicles of *Glycine max* L. under arsenite and arsenate treatments. Each bar represents mean ± Sd of five separate data. Different letters indicate significant differences between treatments ($p < 0.05$).

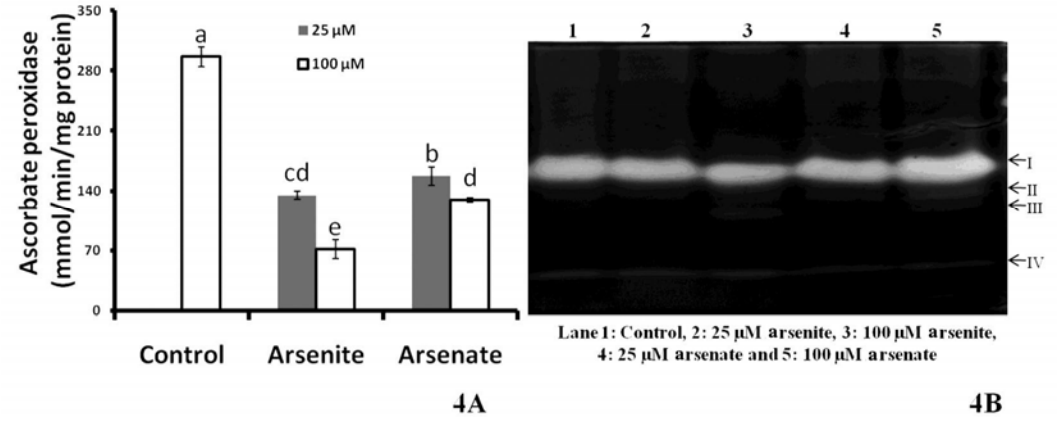

Fig. 4. Biochemical (A) and in-gel (B) activity of ascorbate peroxidase of the *Glycine max* L. radicles exposed to arsenite and arsenate separately. Each bar represents mean ± Sd of five separate determinants. Different letters indicate significant differences between treatments ($p < 0.05$).

Accumulated data suggested that exposure to both the inorganic forms of As (As^{III} and As^{V}) induced oxidative stress in *G. max* L. radicles, but in different intensities. Injury caused by As^{III} was more pronounced than that induced by As^{V}. In addition, *G. max* L. radicles failed to protect itself fully from As-stress which may be due to the insufficient availability of antioxidant components, consequently growth cessation and over accumulation of oxidative stress markers ROS and MDA.

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