ECOPRINT **22:** 7-19, 2015 ISSN 1024-8668
DOI: http://dx.doi.org/10.3126/eco.v22i0.15466
Ecological Society (ECOS), Nepal
www.nepjol.info/index.php/eco; www.ecosnepal.com

STATUS OF ANTIOXIDANT DEFENCE SYSTEM FOR DETOXIFICATION OF ARSENIC IN *BRASSICA JUNCEA* (L.)

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ABSTRACT

The content of arsenic (As), a naturally occurring toxic element found in soils worldwide, has gone substantially high in agricultural soils due to various anthropogenic activities. The responses of seed germination, seedling growth, photosynthetic pigments and the components of the ascorbate-glutathione (AsA-GSH) pathway were analyzed in Indian mustard [*Brassica juncea* (L.) Czern.] cultivar *Pusa Jai Kisan*, treated with 0, 5, 10, 25 and 50 µM As concentrations in a hydroponic system. While a significant increment in lipid peroxidation and H_2O_2 generation was observed at higher concentrations after 2 and 5 days of stress imposition. Significant increases were also observed in superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) activities under the influence of 25 and 50 µM As concentrations after 2 and 5 d stress impositions. There was a steep increase in the ascorbic acid (AsA) content between 2 and 5 d after As treatment, while glutathione (GSH) content increased significantly after 5-d-exposure. Accumulation of As was more in shoot than in root. This study clearly demonstrated that high concentration (50 µM) of As inhibited growth and caused oxidative stress and that the AsA-GSH pathway has an important role in cellular defense against As in *Brassica juncea (*L.).

Key words: Antioxidative enzymes, AsA-GSH pathway, chloroplast pigments, oxidative stress, seed germination.

INTRODUCTION

Contamination of groundwater and soil by arsenic (As), a trace metalloid, is a worldwide problem and poses a potential risks to humans and the environment. As-enriched water is a major source of contamination of the food chain and water supplies (Khan *et al.* 2009).

Efficient, sustainable and environment-friendly ways for removal of As from polluted sites are needed to preserve the quality of drinking water, agricultural products as well as environment. Chaney (1983) was the first to propose the idea of phytoremediation as a 'green' environment-friendly strategy for remediating the metal-contaminated soil and water, in which plants absorb, accumulate and detoxify contaminants from the substrates where they grow through physical, chemical or biological processes (Iqbal *et al.* 2014). The main attribute necessary for metal hyperaccumulation in plants is metal-hypertolerance, which results from an interconnected ensemble of physiological and molecular mechanisms (Diwan *et al.* 2008). Phytoremediation relies mainly on hyperaccumulators, which possess immense ability of heavy-metal accumulation. A model plant for metal cleaning should be rapid in growth, easy in harvesting and capable to tolerate and accumulate large metal concentrations in its shoot (Iqbal *et al.* 2014). Indian mustard [*Brassica juncea* (L.) Czern.], which compensates for its comparatively less metal accumulation by its large yield of shoot dry matter, is recognized with the ability to extract and store metals like Cd, Cu, Ni, Zn, Pb and Se into its shoots (Ansari *et al.* 2015).

A strong antioxidant-defense system is another characteristic that imparts heavy-metal tolerance to plants (Suthar *et al.* 2014). The oxidative stress in plants primarily occurs due to heavy metal-induced generation of free radicals, including toxic reactive oxygen species (ROS) (Ansari *et al.* 2009). However, in order to cope with these ROS-caused anomalies plants have efficient antioxidant defense system that comprises of enzymatic [like superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR)] as well as non-enzymatic [like glutathione (GSH), ascorbate (AsA), carotenoids] components (Khan *et al.* 2009). SOD is the enzyme that catalyzes the dismutation of O_2^- to H_2O_2. The enzymes and metabolites of the AsA–GSH cycle are responsible for the regulation of the H_2O_2 level (Khan *et al.* 2009). The equilibrium between the SOD activity and the activities AsA–GSH cycle enzymes could be critical in deciding the steady-state concentration of O_2^- and H_2O_2. This equilibrium, in addition to the metal-ion sequestration, is thought to be vital for the prevention of ROS formation via the Haber–Weiss or Fenton reactions (Anjum *et al.* 2014).

Despite several works on As-induced changes in plants, information is still deficient on modulation of the AsA-GSH-cycle and changes in growth and physiological attributes of plants under As stress. The main objective of this study was to examine whether the growth characteristics are altered and the antioxidant enzymes are modulated in cultivar *Pusa Jai Kisan* of Indian mustard, when grown under As stress in hydroponic conditions; if yes, whether this can act as a potential As accumulator.

MATERIALS AND METHODS

Plant growth conditions: Indian mustard (*Brassica juncea* L. Czern. cv. *Pusa Jai Kisan*) seeds were procured from the Indian Agricultural Research Institute, New Delhi, India, and germinated on Whatman filter paper soaked in distilled water and kept in the dark for 3 days in a controlled growth chamber at the temperature of 25°C. After germination, these seedlings were shifted to plastic beakers having 250 mL nutrient solution

comprised of 3 mM KNO_3, 2 mM Ca $(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 50 µM KCl, 25 µM H_3BO_3, 2 µM $MnCl_2$, 2 µM $ZnCl_2$, 0.5 µM $CuCl_2$, 0.5 µM $(NH_4)_6MO_7O_{24}$ 20 µM $Na_2FeEDTA$, and 1 mM $MgSO_4$. The pH of the solution was maintained at 6.5 ± 0.1 with 0.1M NaOH. This nutrient solution was aerated continuously with an aeration pump to increase the oxygen level and replaced weekly. The beakers were arranged in a randomized block design and replicated four times. Each beaker had ten plants. After 10 days of growth, plants were treated with 0, 5, 10, 25 and 50 µM of As in the form of Na_3AsO_4. A photosynthetic photon-flux density (PPFD) of 200 µmol m^{-2} s^{-1} was given to the seedlings by combining fluorescent tubes with tungsten lamps, maintaining a photoperiod of 16 h. The plants harvested at 2 and 5 d after As-stress imposition, were rinsed properly with distilled water prior to analysis and measurements. The plant material was separated into roots and shoots (stem + leaves).

Seed germination and arsenic toxicity: Seed germination was tested on wet Whatman (No. 42 mm) filter paper placed in Petri dishes. The paper in Petri dish was moistened with 3.0 mL solution of different As treatments. In the control, the filter paper was moistened with 3.0 mL distilled water. Twenty seeds were placed in each petri dish, which were kept in a growth chamber at 25°C in the dark for 3 days. Each treatment was replicated three times. Germinated seeds were counted on 2 and 5 days after initiation of treatment. The seeds were considered germinated when both plumule and radicle were extended from their junction.

Growth parameters and chloroplast pigments: After obtaining the fresh mass, the plants were dried at 70°C in an oven for determining their dry mass. The root and shoot lengths were measured with a standard scale. The net photosynthetic rate (P_N) of the leaves was calculated with the infrared gas analyzer (IRGA, LI-6400, Lincoln, NE, USA) from the fully expanded uppermost leaves of plants at the maximum irradiance between 11:00–12:00 h. Chlorophyll content of the leaves was estimated by the method of Hiscox and Israelstam (1979).

Assays of antioxidant enzymes: Fresh leaves (0.5 g) were homogenized in a chilled mortar and pestle, using an extraction buffer having 100 mM potassium phosphate (pH 7.0), 0.5% (v:v) Triton X-100 and 1% (w:v) polyvinylpyrrolidone (PVP). The centrifugation of this homogenate was done at 15,000 × g at a temperature of 4°C for 20 min and the enzymes were assayed using the resultant supernatant.

The SOD activity was estimated with the method described by Dhindsa *et al.* (1981) by recording the hinderance of photochemical reduction of nitro-blue tetrazolium (NBT). The enzyme quantity needed for 50% inhibition of the photochemical reduction of NBT was considered as one unit of SOD activity. CAT activity was estimated according to Aebi (1984) by recording the H_2O_2 disappearance and calculated using the extinction coefficient of 0.036 mM^{-1} cm^{-1}. One unit of enzyme referred to quantity of enzyme required for decomposition of 1 µmol of H_2O_2 per min at 25°C. To assay the activity of ascorbate peroxidase (APX), the extraction buffer was supplemented with 2 mM ascorbate. APX activity was estimated by the method of Nakano and Asada (1984) and measured using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. One unit of enzyme was the amount required for decomposition of 1µmol of the substrate per min at 25°C. GR activity was estimated by the method of Foyer and Halliwell (1976) by recording the oxidation of NADPH and using an extinction coefficient of 6.2 mM^{-1} cm^{-1}. One unit of the enzyme refers to the quantity needed for decomposition of 1µmol of NADPH per min at 25°C. Protein content was estimated by the procedure of Bradford (1976), taking the bovine serum albumin (BSA) as a protein standard. The activity of the enzymes was expressed in terms of enzyme units (EU) mg^{-1} protein.

Statistical analysis: The values shown in this work are a mean of four independent observations. A two-way ANOVA test was used for the confirmation of data significance. The comparison with the control and treatments was performed using the Duncan's multiple range tests (DMRT). Changes in antioxidative enzyme activities with As concentration were fit to an exponential rise to the maximum of the form $A_C = A_0 + \Delta A\ (1\text{-}e^{-bC})$, where C was the As concentration in μM and A_C was enzyme activity at concentration C in EU mg^{-1} protein min^{-1}, A_0 was the initial enzyme activity without stress at 0 μM As, ΔA was the maximum increase in enzyme activity equivalent to A_{max}-A_o and b was a fitting parameter determining the shape of the incline. The best-fit parameters were determined using the least squares (Sigma Plot, USA).

Estimation of non-enzymatic antioxidants: Fresh plant tissue (1g) was ground with sand (0.2 g) in 6.5% m-phosphoric acid (5 mL) with 1mM EDTA, using a mortar and pestle. The AsA content was estimated by the method of Hodges *et al*. (1996), and calculated as nmol g^{-1} fresh mass (FM). GSH contents were determined by the method of enzymatic GSSG recycling (Bergmeyer *et al*. 1974). The reaction was monitored spectrophotometrically by stoichiometric conversion of NADPH done at the baseline of NADPH absorbance (340 nm). Standard calibration curve was obtained by using 100 mL of GSSG instead of the sample. One unit mL^{-1} GR was taken to calculate the total glutathione (GSH+GSSG) and GSSG, respectively. The GSH content was obtained by subtracting the GSSG content from the total glutathione and expressed in μmol g^{-1} FM.

Oxidative stress traits: The content of thiobarbuteric acid (TBA) reactive substances, including malondialdehyde (MDA), which are considered to be the products of lipid-peroxidation, were determined in plant samples by the slightly modified method of Heath and Packer (1968). The freshly obtained plant tissue (1 g) was ground in 10 mL of 0.1% TCA (trichloroacetic acid), followed by centrifugation at 10286 × g for 5 min. The supernatant (1 mL) was then combined with 0.5% TBA in 20% TCA (4 mL) in a test tube and subjected to a temperature of 95°C for 30 min, followed by quick cooling in an ice bath and the centrifugation at 2571 × g for 5 min. The absorbance of the supernatant at 532 nm was corrected for unspecific turbidity by subtracting the absorbance at 600 nm. The concentration of lipid peroxidation was expressed as μmol MDA formed g^{-1} FW using an extinction coefficient of 155 mM cm^{-1}.

The H_2O_2 concentration was estimated calorimetrically, as described by Okuda *et al.* (1991). H_2O_2 was extracted by homogenizing plant tissue (0.5 g) with 200 mM perchloric acid (4 mL). The homogenate was subjected to centrifugation of 12,000 × g for 10 min. H_2O_2-induced oxidation of ferrous ions to ferric ions in acidic pH was monitored and the stable complex of ferric ions with xylenol orange dye was measured at 560 nm. The H_2O_2 content was shown in nmol g^{-1} FW.

Elemental analysis: The As contents in roots and shoots were calculated after the samples were subjected to digestion in sulphuric/nitric acid mixture (2:1, v/v). The harvested plant material was washed thoroughly with Milli-Q water (resistivity >18 MΩ cm at 25°C), dried at 65°C ± 2°C for 72 h, and digested after grinding to fine powder, using the method of Ansari *et al.* (2015). In brief, dry material (0.25 g) from each treatment sample was added to concentrated HNO_3 (3 mL) in a 50 ml digestion tube and swirled gently for mixing. These digestion tubes were kept for 1 h at 150°C in a heating block set. Subsequently, the tubes were cooled. Afterwards, 2 mL of 30% H_2O_2

was put in each digestion tube and heated for an additional 3 h at 150°C. Finally, the tubes were cooled down to room temperature. After the plant tissues were completely digested, the solution was diluted to 50 mL and the supernatant analyzed for of As content, using an atomic absorption spectrophotometer (ZEEnit 65, Analytik Jena, Germany) fitted with a graphite tube of the Wall type (Ansari *et al.* 2015).

RESULTS AND DISCUSSION

Seed germination: Data obtained on seed germination indicated that As was highly toxic to the genotype studied. Seed-germination rate declined significantly with increase in As concentration applied. The reduction in seed germination, which varied day wise, was in the range of 8–40% on the 2nd day and 12-60% on the 5th day of experiment, as compared with the control (Fig. 1). Thus, the reduction was highest and most significant on the 5th day. Our results indicate that seed germination was minimized significantly with increase As treatments, as recorded on the 2nd and the 5th day of treatment. Similar effects of As on seed germination have been observed in wheat, rice, and green gram (Mumthas *et al.* 2010). Seed germination relies almost solely on seed reserves of metabolites available for meeting the requirement of respiration other anabolic reactions. The starch is quantitatively the most copious storage material in the seeds, and the available evidence suggests that in germinating seeds starch is degraded predominantly via the amylolytic pathway (Mumthas *et al.* 2010). Seed coat provides protection from metal stress prior to germination, but it cracks finally crack or becomes more permeable upon germination. It is known that seed germination is affected by metals or metalloid due to their toxicity that causes various nutritional disturbances and also suppression of water uptake. Thus, metal treatment normally causes a concentration-dependent diminution in seed germination, as observed in a number of species (Lamhamdi *et al.* 2011).

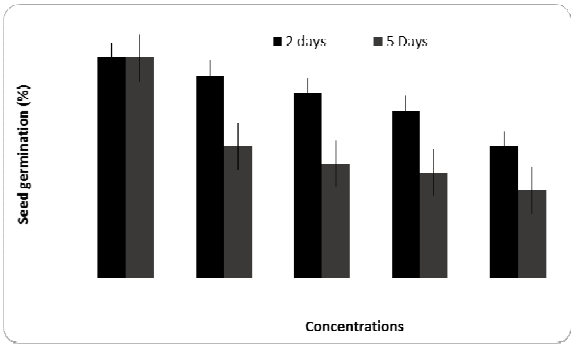

Fig. 1. Seed germination percentage of 2 & 5 day old *Brassica juncea* cv. *Pusa Jai Kisan* grown with and without As stress. All values are the mean of four independent experiments with four replications each (n = 12). Significant ($p \leq 0.05$) compared to control. The percentage error in replicate reproducibility was < 2% in all cases.

Growth variables: The mild dose of As (5 µM) did not affect the biomass, root and shoot lengths, photosynthesis and chlorophyll content in the species studied. However, a gradual decrease in these parameters was observed with increasing As concentration in the medium (Table 1). Relative to the controls, the maximum decrease occurred at 50 µM, showing a variation of 46.1, 44.7, 18.5, 27.0, 25.1 and 66.8%, for root mass, shoot mass, root length, shoot length, net photosynthetic rate and chlorophyll content respectively. At this concentration, As accumulation in the shoot was much higher (263.3 µg As g^{-1} DW) than in roots (134.6 µg As g^{-1} DW), indicating a tendency for metal retention preferably in the aerial plant parts (Fig. 2). The low (5 µM) As treatment did not influence the root and shoot growth significantly,

which suggests that *Pusa Jai Kisan* is tolerant for As at low doses. Nevertheless, higher doses (25 and 50 µM) were toxic and caused significant reductions. Khan *et al.* (2009) reported a significant reduction in growth parameters of this plant at 25 µM As exposure. Our results also showed a substantial diminution in growth parameters at 25 µM dose. Growth inhibition at higher concentrations may be linked with lower mitotic activity in the root meristematic zone or inhibition of cell enlargement in the elongation zone as a consequence of decreased cellular turgor (Mumthas *et al.* 2010), as root lengthening is controlled by the cell-division rate in the apical meristems and by expansion and elongation of the newly formed cells. A dose-dependent inhibition of root growth (and of the whole plant), has been demonstrated in wheat, mung bean, arabidopsis, broad bean and rice (Ansari *et al.* 2013). Moreover, the higher HM build-up in plant tissues may affect the energy balance within the plant because plants would use energy to survive these high metal concentrations (Greger 1999), by activating production of antioxidants and phytochelatins and this would hamper photosynthesis, leading to decline in growth (Cao *et al.* 2009).

Arsenic is known to inhibit biomass production in various plant species (Srivastava *et al.* 2005), which might be due to increased tissue permeability and tissue loss, inhibition of cell division, reduced enzyme activity and/or the As-induced oxidative stress (Xiao *et al.* 2011).

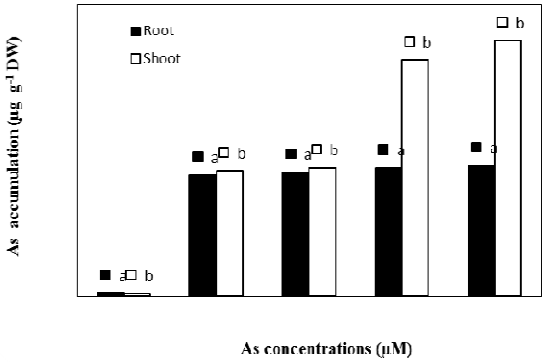

Fig. 2. As accumulation in the root and shoot of 20 day old *Brassica juncea* cv. *Pusa Jai Kisan* grown with and without As stress. All values are the mean of four independent experiments with four replications each (n = 12). Significant ($p \leq 0.05$) compared to control. Error bars are not shown as they are insignificant, the percentage error in replicate reproducibility was < 2% in all cases.

Table 1. Plant growth parameters of *Brassica juncea*. cv *Pusa Jai Kisan* as influenced by as treatment, observed in 20 day old plants.

Parameters	As concentration (µM)				
	0	5	10	25	50
Root dry mass (g plant^{-1})	0.13 ± 0.03^{a}	0.12 ± 0.03^{b}	0.11 ± 0.04^{c}	0.10 ± 0.03^{d}	0.07 ± 0.03^{e}
Shoot dry mass (g plant^{-1})	0.38 ± 0.05^{a}	0.37 ± 0.04^{b}	0.31 ± 0.02^{c}	0.30 ± 0.05^{cd}	0.21 ± 0.06^{e}
Root length (cm)	11.4 ± 0.3^{a}	11.0 ± 0.3^{b}	10.0 ± 0.01^{bc}	9.4 ± 0.3^{d}	9.3 ± 0.4^{e}
Shoot length (cm)	14.8 ± 0.3^{a}	14.7 ± 0.3^{b}	12.8 ± 0.3^{c}	11.8 ± 0.3^{d}	10.8 ± 0.3^{e}
Photosynthetic rate (µmol CO_2 m^{-2} sec^{-1})	13.7 ± 0.3^{a}	13.6 ± 0.03^{b}	11.9 ± 0.1^{c}	11.1 ± 0.1^{cd}	10.3 ± 0.06^{e}
Chlorophyll content (mg g^{-1} FW)	1.6 ± 0.1^{a}	1.4 ± 0.02^{b}	0.95 ± 0.03^{c}	0.76 ± 0.02^{d}	0.52 ± 0.03^{e}

All values are the mean of four independent experiments having four replicates in each experiment (n = 12). Means in a row marked with similar letters do not differ significantly ($p < 0.05$)

Photosynthetic pigments: Since photosynthetic activity is intimately correlated to the quantity of chloroplast pigment, as observed in this as well as earlier studies on different plants such as chickpea (Srivastava *et al.* 2014), tomato and rice seedlings (Choudhary *et al.* 2010), and because these pigments are among the main receptors of metal toxicity (Miteva and Merakchiyska 2002), the resultant inhibition of chlorophyll synthesis becomes the limiting factor for photosynthesis. In metal-stressed plants, Mg ion in the chlorophyll molecule is usually substituted with the available heavy metal under low-light conditions, which markedly disturbs the photosynthetic activity (Srivastava *et al.* 2014). Furthermore, the reduction in chlorophyll content in plants exposed to heavy-metal stress may be due to (a) inhibition of important enzymes, such as δ-aminolevulinic acid dehydratase (ALA-dehydratase) and protochlorophyllide reductase (John *et al.* 2008), associated with chlorophyll biosynthesis, (b) impairment in the supply of Mg^{2+} and Fe^{2+} required for the synthesis of chlorophylls, (c) Zn^{2+} deficiency resulting in inhibition of enzymes such as carbonic anhydrase, and (d) replacement of Mg^{2+} ions associated with the tetrapyrrole ring of the chlorophyll molecule (John *et al.* 2008).

Arsenic accumulation: Heavy-metal accumulation in higher plants stimulates a number of intracellular changes and few of them contribute directly to the metal tolerance capability in plants (Sinha *et al.* 2007). In our study, greater As accumulation in shoots (243.3 µg As g^{-1} DM) than in roots (134.6 µg As g^{-1} DM) at 20 DAT at higher (50 µM) dose (Fig. 2) implies that a considerable amount of As was translocated to aerial plant parts (Ansari *et al.* 2013). Gupta *et al.* (2009) asserted that As accumulation was more in roots than in shoots of Indian mustard after a short-term (2d) exposure, but it was more in shoots than in roots after a longer (5d) exposure, possibly due to involvement of As transporters in the long-distance (root to shoot) metal translocation.

Lipid peroxidation levels, expressed in terms of MDA and H_2O_2 contents, increased with increase in As concentration in the medium (Fig. 3). The most significant increase in lipid peroxidation at the highest concentration (50 µM), showing an increase of 46.2% in MDA and 63.1% in H_2O_2 contents, over the control. Generally, these increases in MDA and H_2O_2 contents were linearly correlated ($r^2 > 0.97$) with the increase in As concentrations (Fig. 3). In addition, slopes of the curves for 2 and 5 DAT samples were generally similar within the experimental error, indicating that the relative changes in MDA and H_2O_2 contents were similar regardless of the time of analysis, with the 5 DAT results being consistently higher by a predefined amount equal to 2.3 and 1.4 µmol g^{-1} FW for MDA and H_2O_2, respectively. Plant responses to metals are complex and may involve several different simultaneous defense mechanisms to withstand metal toxicity. However, heavy metals primarily damage the molecular structure in plant cells either directly or indirectly through formation of reactive oxygen species (ROS) (Iqbal *et al.* 2014), including free radical and non-radical molecules of high reactivity, like H_2O_2 and singlet oxygen ($^1O_2$). Membrane lipids are particularly susceptible to free radical attack. Protonation of the superoxide radical produces hydroperoxyl radicals (^{-}OH, H_2O_2), which change fatty acids into toxic lipid peroxides, damaging the biological membranes (Anjum *et al.* 2014). Active oxygen radicals may trigger the continuous unsaturated membrane-fatty-acid peroxidation in producing the lipid-peroxidation products, like MDA (Mishra *et al.* 2008). Since lipid peroxidation is attributed to oxidative damage, the levels of MDA, a usual product of lipid peroxidation, can be recruited as a potential indicator of oxidative stress (Suthar *et al.* 2014). In our study, both the MDA contents and H_2O_2 production increased linearly with increased As concentrations (Fig. 3). Consequently, the MDA and H_2O_2 levels in treated samples were significantly greater than in the controls, this being a strong evidence of As-induced oxidative stress in

cv. *Pusa Jai Kisan.* In a previous study, Khan *et al.* (2009) demonstrated that a short-term (< 4 days) As exposure of this species did not significantly affect the antioxidant metabolism. Therefore, in the current study, the exposure period was increased to 10 days to examine the longer-term exposure effect.

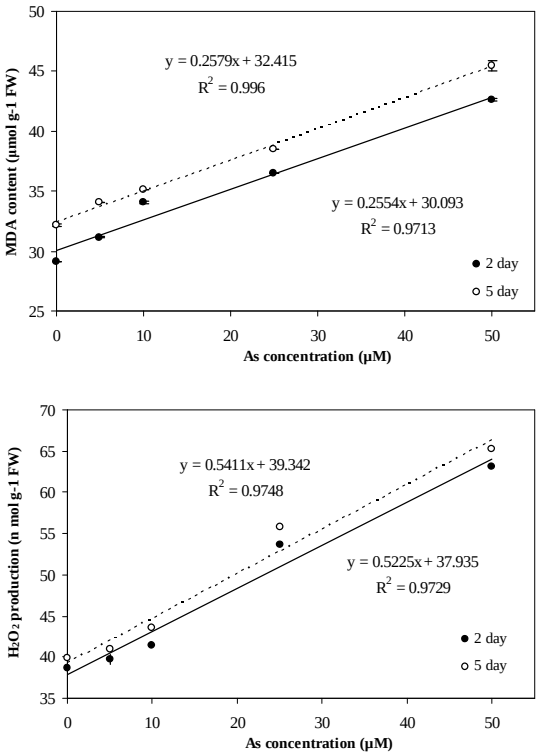

Fig. 3. MDA (a) and H_2O_2 contents (b) in the leaves of 2 & 5 day old *Brassica juncea* cv. *Pusa Jai Kisan* grown at various levels of As. All values are the mean of four independent experiments with four replications each (n = 12). Significant ($p \leq 0.05$) compared to control. Solid and dashed lines correspond to simple linear fits of the data at 2 and 5 d, respectively. Error bars are not shown as they are insignificant, the percentage error in replicate reproducibility was < 2% in all cases.

Antioxidative enzymes: Enzyme activities of SOD, APX, CAT and GR were analysed in the leaves of Indian mustard plants subjected to different As concentrations (Fig. 4). These enzymes showed increased activity with increase in As concentration in the medium. The SOD and CAT activities increased by 65.8 and 28.5% at 2d and by 49.6 and 35.5% at 5d, respectively, as compared with their controls. However, the APX and GR activities increased by 49.5 and 150% after 2-day treatment and by 40.1 and 187.5% after 5-day treatment, respectively (Fig. 4). These differences showed significant at $p \leq 0.05$. The activities of the antioxidant enzymes were fit with good correlations ($r^2 > 0.95$) by a rise to the maximum curve (Table 2). The observed activities were consistently higher at 5d exposure than at 2d exposure; while the shapes of the curves at 2 and 5d were similar for SOD and APX, it was not so for CAT and GR, which showed a slightly greater activity for 5-day exposure than for 2-day exposure. The SOD is one of the most vital antioxidant enzymes defending plants against the ROS-mediated toxicity by catalyzing the superoxide radical dismutation to hydrogen peroxide and molecular oxygen (Khan *et al.* 2009). Gupta *et al.* (2009) observed that As induced a strong antioxidative defense at low (< 150 µM) As concentration, which declined at higher (300 µM) concentrations in two different cultivars of Indian mustard. In the present study, the As concentrations were much lower than those required to cause SOD inactivation and, consequently, the SOD activity was significantly greater in the leaves of *B. juncea* at both the minimum (5 µM) and maximum (50 µM) As doses in comparison with the controls. Since SOD activity produces H_2O_2, which again is toxic for cells and needs further detoxification by CAT in the AsA-GSH cycle, CAT is another important enzyme involved in the process to dismutate H_2O_2

and decompose it to H_2O and O_2 (Khan *et al.* 2009). We found that involvement of CAT was insignificant in active H_2O_2 reduction regardless of the dose of As. Similar results for CAT activity were observed previously under other abiotic stresses, which could be because of inhibited enzyme synthesis or a changed assembly of enzyme subunits, as suggested by Ogawa *et al.* (1997). The AsA-GSH cycle in chloroplasts mainly involves APX and GR enzymes. In our experiment, APX activity was significantly enhanced with increase in the As dose and the duration of exposure. The enhanced APX activity provides a clue of the role of APX in the dismutation of H_2O_2 and its up-regulation in As-induced oxidative stress.

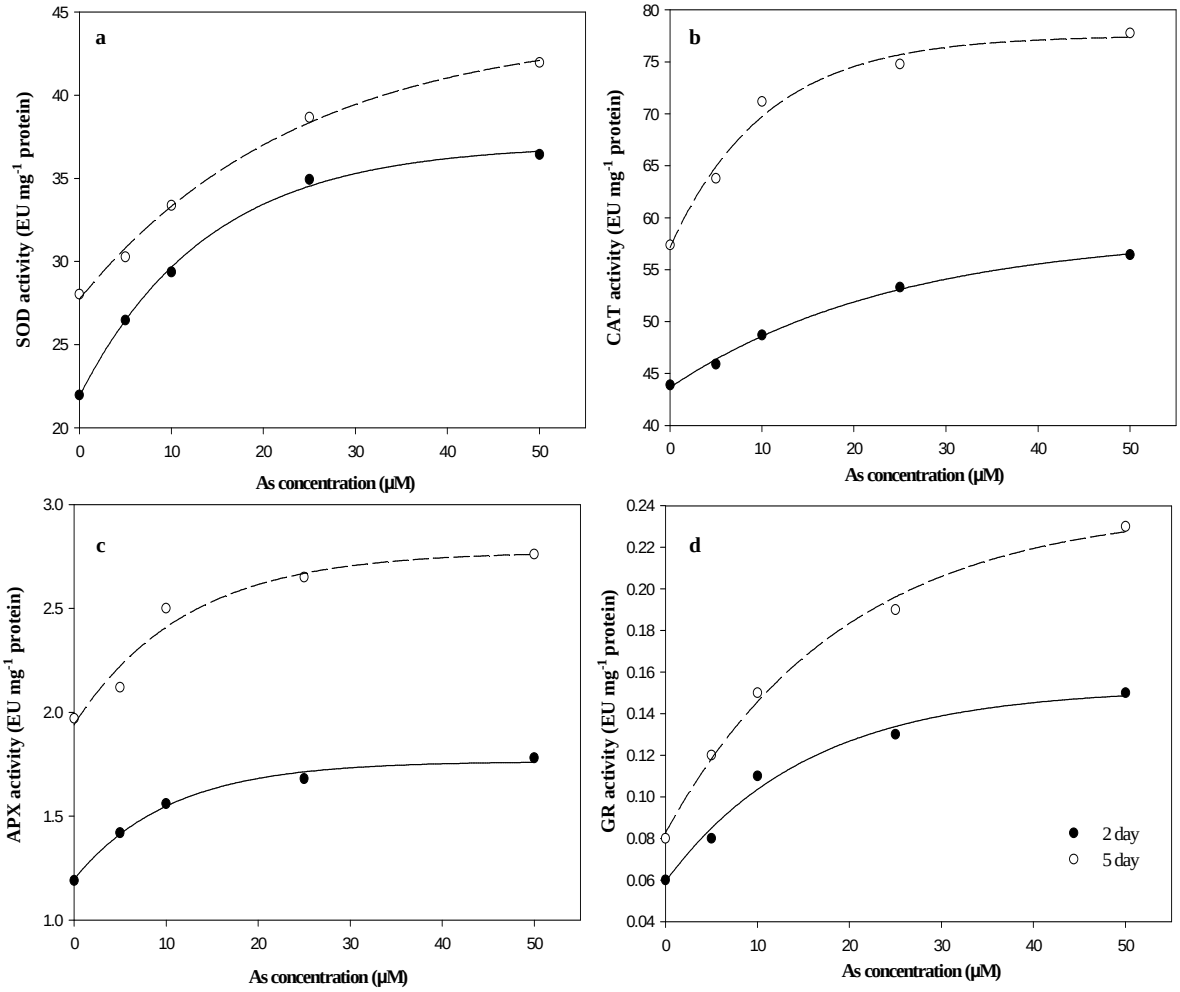

Fig. 4. Activities of SOD (a), CAT (b), APX (c) and GR (d) in 2 and 5 day old *Brassica juncea* cv. *Pusa Jai Kisan* grown with and without As stress. All values are the mean of four independent experiments with four replications each (n = 12). Significant ($p \leq 0.05$) compared to control.

Table 2. Best fit parameters for enzyme activities of SOD, CAT, APX and GR at 2 and 5 d due to stress induced by increased as concentration.

Enzyme Monitored	Best Fit Parameters			
	A_0	ΔA	B	r^2
SOD – 2 day	21.9 ± 0.3	15.2 ± 0.5	0.072 ± 0.006	0.998
SOD – 5 day	27.7 ± 0.4	16.4 ± 1.1	0.042 ± 0.007	0.996
CAT – 2 day	43.6 ± 0.3	14.8 ± 0.9	0.041 ± 0.006	0.997
CAT – 5 day	57.1 ± 1.4	20.4 ± 1.8	0.09 ± 0.02	0.984
APX – 2 day	1.20 ± 0.02	0.57 ± 0.03	0.10 ± 0.01	0.992
APX – 5 day	1.94 ± 0.09	0.8 ± 0.1	0.08 ± 0.03	0.957
GR – 2 day	0.059 ± 0.006	0.093 ± 0.009	0.06 ± 0.01	0.984
GR – 5 day	0.083 ± 0.005	0.16 ± 0.01	0.052 ± 0.009	0.995

Significant increases ($p \leq 0.05$) were recorded in AsA contents of the leaves of As-treated plants till 5d of metal exposure and a comparatively steady level thereafter (Fig. 5a). In comparison with the control, there was a sharp enhancement of 19.6% and 41.0% in the AsA contents with the low and high As treatments, respectively. Accumulation of GSH in the leaves of As-exposed plants was recorded from 2d of exposure onwards and was significant ($p \leq 0.05$) relative to the control (Fig. 5b). The level of ascorbate (AsA), a major reducing substrate for H_2O_2 detoxification (Anjum *et al.* 2008), significantly increased with As treatments, as compared with the controls. DHA is formed as a result of reduction of AsA and is recycled back to AsA, using the GSH as an electron donor. GR is a key enzyme involved in the reduction of GSSG to GSH through oxidation of NAD (P) H to NAD (P) $^+$. Since the oxidized glutathione (GSSG) is converted back to GSH with the help of GR, the GSH witnesses a concentration as well as time-dependent enhancement in the As-exposed plants. Accordingly, an increased GSH concentration corresponds with the capacity of plants to combat the oxidative stress caused by metals. In a recent study, Indian mustard plants exposed to 0.0, 0.1, 0.2 and 0.3 mM concentration of arsenic (V) and harvested after 30 and 60 days of sowing showed

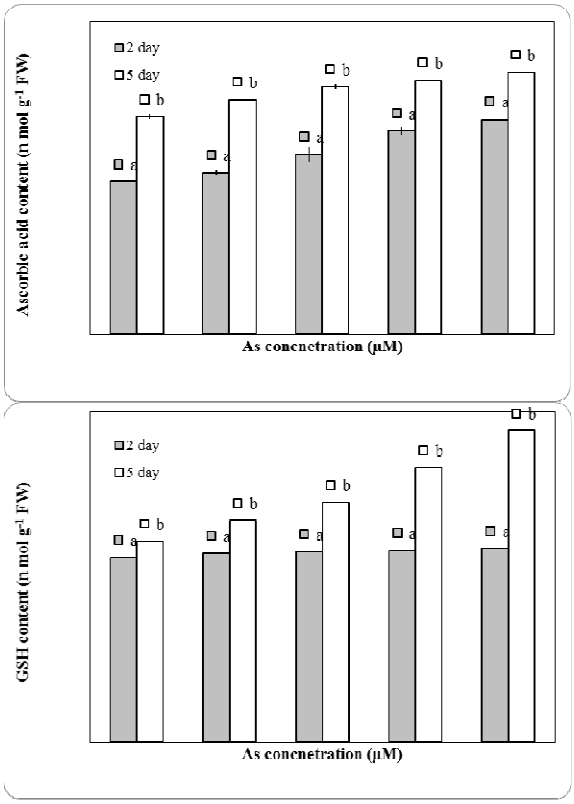

Fig. 5. Contents of AsA (a) and GSH (b) in leaves of 2 & 5 day old *Brassica juncea* cv. *Pusa Jai Kisan* grown with and without As stress. All values are the mean of four independent experiments with four replications each (n = 12). Error bars are one standard deviation of the mean. Significant ($p \leq 0.05$) compared to control.

that As significantly hampered the growth variables, triggered the modulations of various stress markers like proteins, antioxidant enzymes, and MDA content, and induced the synthesis of four brassinosteroids, namely castasterone, teasterone, 24-epibrassinolide, and typhasterol (Kanwar *et al.* 2015). In the present study, GR activity showed a considerable increase under the influence of As treatments, being the maximum with the highest As doses, indicating that, on the whole, the AsA-GSH cycle in *Brassica juncea* was quite capable of detoxifying the reactive oxygen species.

CONCLUSIONS

As treatments induced oxidative stress, as demonstrated by enhanced lipid peroxidation and H_2O_2 levels cv. *Pusa Jai Kisan* of Indian mustard. However, to withstand the As toxicity, it developed a defence strategy through modulation of enzymatic and non-enzymatic antioxidant components to prevent the expected cellular damage. This could successfully nullify the deleterious effects and helped in maintaining plant growth at low As concentration. In conclusion, *Pusa Jai Kisan* is a suitable As phytoremediator due to its high As-accumulation capacity and a sound antioxidant defense system. The As content in treated plants increased linearly with increase in the As levels applied, with As accumulation being higher in shoots than in roots.

ACKNOWLEDGEMENTS

The first author thanks the Hamdard National Foundation, New Delhi, for financial assistance for this study. Mohd Kafeel Ahamd Ansari was a Visiting Scientist at the University of South Australia during the development of this manuscript. Last co-author gratefully acknowledges the financial support of the Australian Research Council Future Fellowship Scheme (grant number: FT120100799) for funding his salary.

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