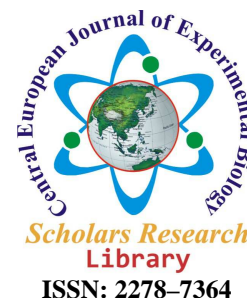




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## Arsenic-induced oxidative stress and its reversal by thiourea in mung bean (*Vigna radiata* (L.) Wilczek.) genotype

Dibyendu Talukdar

Department of Botany, R.P.M. College (University of Calcutta), Uttarpara, Hooghly, west Bengal

### ABSTRACT

A pot culture experiment was conducted in mung bean (*Vigna radiata* (L.) Wilczek.) genotype PUSA Baisakhi to test the effect of sodium arsenate and thiourea (TU). Arsenic at 25  $\mu\text{M}$  caused significant inhibition of growth as indicated by reduced shoot and root dry weight and reduction in photosynthetic capacity. Significant decline in ascorbate peroxidase, dehydroascorbate reductase and glutathione reductase coupled with high superoxide dismutase seriously impeded scavenging of reactive oxygen species and led to reduction in glutathione redox state. This resulted in overaccumulation of  $\text{H}_2\text{O}_2$  in leaves of arsenate-treated mung bean genotype and consequently, high level of lipid peroxidations, as the marks of onset of arsenic-induced oxidative stress. Priming of seeds and application of TU at 6.5mM and 13 mM was found highly effective not only in countering of oxidative stress but also in significant enhancement of shoot and root dry weight even under As exposure. Significant elevation in  $\text{H}_2\text{O}_2$ -scavenging capacity accompanied by high GSH-regeneration though stimulated glutathione reductase activity in presence of TU certainly helped mung bean seedlings to counter As-induced oxidative stress and maintenance of normal to even better plant growth and photosynthesis under As exposure.

**Key words:** Antioxidant defense, arsenate, mung bean, oxidative stress, thiourea

### INTRODUCTION

Mung bean [*Vigna radiata* (L.) Wilczek] also known as green gram, is one of the important grain legumes of India. Seeds are rich in digestible protein (approximately 25–28 %). It is extensively grown in tropical and subtropical Asia because of its wider range of adaptability, fitting well in multi-cropping systems [1]. In India, mung bean is grown in two seasons: during summer and winter. However, its large-scale adoption is affected by low yield potential accompanied with various biotic and abiotic factors [2,3].

Arsenic (As) is a wide-spread toxic metalloid, constituting the biggest abiotic stress factors for mankind. Groundwater contamination by As and its entry into crops through water-soil-plant system has caused great environmental concern. The bioaccumulation of As in crop plants has huge negative impact for public health issues [4], and this is of great environmental concern because As is known to be a carcinogen and a powerful co-mutagen [5,6]. Besides rice and vegetables, quite alarmingly, As is gradually entering into pulse food system through its accumulation in major legumes like chickpea, pea, lentil, beans, fenugreek and grasspea [7-13]. As can induce severe oxidative stress in legume plants through alteration in cellular and metabolic redox homeostasis, severely jeopardizing plant growth and inflicting damage to yield and nutritional quality of grain, and may also render the crops vulnerable to other biotic and abiotic stresses [8,10, 14-16]. Being grown in aerobic fields, legumes are

exposed to arsenate forms of As which may either directly or through conversion to highly toxic arsenite ( $\text{As}^{\text{III}}$ ) adversely affects plant growth by generating excess ROS and consequent oxidative damage to lipids and proteins through alterations in antioxidant defense comprising of non-enzymatic components, ascorbate-glutathione cycle enzymes, and components outside this cycle [14, 15, 17]. Accumulating evidences indicate that As exposure seriously jeopardizes plant thiol-redox status, and the glutathione (GSH) and GSH-mediated antioxidant defense plays key roles in determining As-tolerance in different plants [18-21]. As-induced oxidative damage and consequent growth inhibition has primarily been reported in chickpea, pea, grass pea, lentils, common beans, and fenugreek [8-12, 14-16]. In mung bean, As-induced oxidative stress was attributed to oxidative damage to membrane and consequent inhibition of plant growth [22]. In the background of As-toxicity in edible food legumes, development of safe crop with high yield and nutritional quality in contaminated soil may be one of the important strategies to counter the detrimental impacts of As [20, 21] for which understanding of mechanistic details of As-induced stress and inducible tolerance is extremely important.

Grain priming and foliar pretreatment has been achieved progressively in many plant species and is potent in improving yield quality and amounts [23, 24]. Among the mitigation strategies and improving crop productivity under stressful environments, use of sulfhydryl bio-regulator like thiourea (TU), a ROS-scavenger, has been found highly effective in ameliorating salt as well as UV stress in *Brassica juncea*, and maize, and in tuberization of potato through alterations in antioxidant defense and/or sink translocations and modulations of gene expressions at transcriptional levels [25-29]. The concept behind the use of TU is based upon the fact that it is a non-transgenic approach and acts as Sulfur-containing defense compounds during adaptations of plants to biotic and abiotic stress by strengthening the plant's *built-in* mechanisms through priming mediated physiological and molecular tuning. TU was primarily found to increase productivity in mung bean [30], but despite high sensitivity of mung bean to As-stress, no study, is carried out to test the potential of TU in inducing tolerance to As-induced oxidative stress in mung bean. The present study was therefore carried out to reveal the potential of TU in inducing tolerance of mung bean to As-toxicity through assessing alterations in morpho-physiological and biochemical characteristics.

## MATERIALS AND METHODS

### *Plant materials, treatment protocols and culture conditions*

Fresh and healthy seeds of mung bean (*Vigna radiata* (L.) Wilczek, cv. PUSA Baisakhi) were surface-sterilized with NaOCl (0.1 %, w/v), continuously washed under running tap water followed by distilled water, and were allowed to germinate in the dark in two separate sets on moistened filter paper at 25 °C. Germinated seedlings were immediately placed in polythene pots (8 plants pots<sup>-1</sup>) containing 300 ml of Hoagland's No 2 nutrient media following earlier protocol [31], and were allowed to grow for 10 d. The plants were, then, subjected to the following treatment protocols as: (a) untreated control, (b) 20  $\mu\text{M}$  sodium arsenate (As, MW 312.01 g/mol; technical grade, purity 98.5 %, Sigma-Aldrich), (c) 25  $\mu\text{M}$  As+ 6.5 mM thiourea (TU), and (d) 25  $\mu\text{M}$  As+13 mM TU. Each treatment was replicated four times. TU (Sigma-Aldrich, Bangalore, India), a sulfhydryl bio-regulator, was used to presoak the seeds and then in foliar spray in the last two protocols. Pilot experiments were carried out to determine the effective doses of TU and As without causing toxicity to seedlings. Control and treated plants were allowed to grow for another 10 d. Nutrient solution was refreshed every alternate day to prevent depletion of nutrients, TU as well as As in the course of the plant's exposure to the metalloid. The experiment was carried out in a completely randomized block design manner in an environmentally controlled growth chamber under a 14 h photoperiod, 28/18 ( $\pm 2$  °C), relative humidity of  $70 \pm 2$  % and a photosynthesis photon flux density of 200  $\mu\text{molm}^{-2}\text{s}^{-1}$ . Plants were harvested after stipulated period, parts were separated, thoroughly washed and oven dried at 72 °C for 48 h to take dry weights of shoots and roots.

### *Determination of chlorophyll and total carotenoids*

Leaf chlorophyll (Chl) and carotenoid contents were determined by the method of Lichtenthaler [32]. Leaf tissue (50 mg) was homogenized in 10 ml chilled acetone (80 %). The homogenate was centrifuged at 4,000 g for 12 min. Absorbance of the supernatant was recorded at 663, 647 and 470 nm for chlorophyll a, chlorophyll b and carotenoids, respectively. The contents were expressed as mg chl or carotenoids g<sup>-1</sup> fresh weight (FW).

### *Assay of antioxidant enzyme activity*

Fresh leaf tissue of 250 mg was homogenized in 1 ml of 50 mM K-phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol and 2 % (w/v) polyvinyl pyrrolidone (PVP) using a chilled mortar and pestle kept in an ice bath. The homogenate was centrifuged at 15,000g at 4 °C for 20 min. Clear supernatant was used for enzyme

assays. For measuring APX activity, the tissue was separately ground in homogenizing medium containing 2.0 mM AsA in addition to the other ingredients. All assays were done at 25 °C, as detailed earlier [31,33, 34]. Soluble protein content was determined using Bovine Serum Albumin as standard [35].

SOD (EC 1.15.1.1) activity was determined by nitro blue tetrazolium (NBT) photochemical assay [36] and was expressed as U (unit)  $\text{min}^{-1} \text{mg}^{-1}$  protein. One unit of SOD was equal to that amount causing a 50 % decrease in SOD-inhibited NBT reduction. APX (EC 1.11.1.11) activity (nmol AsA oxidized  $\text{min}^{-1} \text{mg}^{-1}$  protein) was assayed following Nakano and Asada [37] with  $\text{H}_2\text{O}_2$ -dependent oxidation of AsA followed by a decrease in the absorbance at 290 nm ( $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ). DHAR (EC 1.8.5.1) activity was measured following the protocol of Nakano and Asada [37]. Enzyme activity was expressed as  $\mu\text{mol}$  ascorbate formed  $\text{min}^{-1} \text{mg}^{-1}$  protein. Glutathione reductase (GR, EC 1.6.4.2) activity was determined by monitoring the glutathione dependant oxidation of NADPH, as described by Carlberg and Mannervik [38]. Reaction was initiated by adding 0.1 ml enzyme extract to the cuvette and the decrease in absorbance at 340 nm was monitored for 2 min. GR specific activity was expressed as nmol NADPH oxidized  $\text{min}^{-1} \text{mg}^{-1}$  protein. CAT (EC 1.11.1.6)-specific activity (nmol  $\text{H}_2\text{O}_2$  degraded  $\text{min}^{-1} \text{mg}^{-1}$  protein) was calculated using the molar absorptivity of  $43.6 \text{ M}^{-1} \text{ cm}^{-1}$  for  $\text{H}_2\text{O}_2$  at 240 nm [39]. Foliar total and reduced glutathione (GSH) was estimated following Griffith [40]. GSH redox was calculated as  $[\text{GSH}/(\text{GSH}+\text{GSSG})]$ .

#### *Determination of $\text{H}_2\text{O}_2$ Content and lipid peroxidation level*

Fresh leaf tissue of 0.1 g was powdered with liquid nitrogen and blended with 3 ml acetone for 30 min at 4 °C. Then, the sample was filtered through eight layers of gauze cloth. After the addition of 0.15 g of active carbon, the sample was centrifuged twice at 3,000 g for 20 min at 4 °C, and then 0.2 ml 20 %  $\text{TiCl}_4$  in HCl and 0.2 ml ammonia were added to 1 ml of the supernatant. After reaction, the compound was centrifuged at 3,000g for 10 min, the supernatant was discarded, and the pellet was dissolved in 3 ml of 1 M  $\text{H}_2\text{SO}_4$ .  $\text{H}_2\text{O}_2$  content was measured from the absorbance at 410 nm using a standard curve, following Wang et al. [41]. Lipid peroxidation rate was determined by measuring the malondialdehyde (MDA) equivalents following Hodges et al. [42] and was expressed as nmol MDA  $\text{g}^{-1} \text{FW}$ .

#### *Statistical analysis*

The results are the mean values  $\pm$  standard errors of at least four replicates. Multiple comparisons of means were performed by ANOVA (SPSS Inc. v. 10), and the means were separated by Duncan's multiple range test with significance level at  $P < 0.05$ .

## RESULTS AND DISCUSSION

Mung bean genotype PUSA Baisakhi exhibited severe growth inhibition as manifested by significantly reduced shoot and root dry weight under 25  $\mu\text{M}$  As exposure. Compared to control, shoot dry weight was reduced by 2-fold whereas root dry weight was reduced by about 3.5-fold (Table 1). Decrease in seed germination and length of plant parts was observed as concentration dependent way in other mung bean genotypes subjected to arsenic tri-oxide solution [43]. Inhibition of biomass production was also observed in edible legumes like common beans, lentils, grass pea and chick pea under arsenic exposures [8-12, 14-17]. Growth inhibition was might be due to significant reduction in plant photosynthetic capacity as manifested by significant reduction in chl a, chl a/b ratio and total carotenoid contents in the present mung bean genotypes. Inhibition of photosynthesis has been considered one of the single most factor for reduction of plant growth and yield in different plants exposed to arsenic [14, 16, 17].

Significant reduction of growth traits and photosynthetic apparatus was accompanied with severe alterations in primary antioxidant defense capabilities in mung bean genotypes under As treatment. Compared to control, SOD activity was increased by about 3-fold whereas activities of APX, DHAR and GR were reduced by nearly 4-, 3.2, and 2-fold, respectively (Table 1). SOD constitutes the first line of defense against reactive oxygen species (ROS) [44]. Thus, increase in its activity in As-treated mung bean genotype indicates generations of excess superoxide radicals under As-exposures and increased dismutation to counter it. However, SOD activity generates  $\text{H}_2\text{O}_2$  as byproducts of dismutations [44].  $\text{H}_2\text{O}_2$  is a prominent ROS in plant cell and can diffuse to damage cellular constituents particularly when thiol-containing enzymes are functioning. The dual roles of  $\text{H}_2\text{O}_2$  as a signaling molecule for stress perception and an inducer of oxidative stress has been well documented in *Arabidopsis* and in several crop plants like cereals, and legumes [33, 45,46]. Within ascorbate-glutathione cycle, APX is the most prolific enzymes to scavenge  $\text{H}_2\text{O}_2$ , using ascorbate as an exclusive co-factor [44]. Low DHAR activity may jeopardize the regeneration of reduced ascorbate from dehydroascorbate, generated by APX action, while decreased

GR level may hamper regeneration of reduced glutathione (GSH) from its oxidized form, the GSSG. GSH is the most important low-molecular weight thiol buffer within plant cells, participate in numerous cellular and metabolic functions in plants [44, 47, 48]. Declining redox state of GSH in the leaves of present mung bean seedling under As-exposure was mainly due to decrease in GSH level (Table 1) which coupled with low APX might be responsible for enhanced ROS generation in leaves, despite the fact that CAT activity was as per the control leaves (Table 1). Certainly, mung bean seedlings suffered oxidative stress as rising H<sub>2</sub>O<sub>2</sub> level may trigger membrane lipid peroxidation, as evidenced by significantly higher MDA content in treated mung bean leaves (Table 1).

Use of TU at two different concentrations had significant reversal of As-induced growth inhibition in mung bean seedlings. While 6.5mM was found effective to counter the growth reduction, use of 13 mM TU was highly effective to enhance plant growth. Both shoot and root dry weights were very close to control at 6.5 mM TU + As treatment but even significantly higher than control values in 13 mM TU + As treatment (Table 1). Leaf chl a, chl a/b ratio and total carotenoids were also marginally varied in relation to control (Table 1). TU application was found highly effective in improvement of plant photosynthesis, growth, and yield in different crop plants such as mung bean, wheat, Brassica, and maize. In *Brassica juncea*, TU application improved the plant growth potential, photosynthetic efficiency, and yield which was attributed to capacity of TU to maintain redox homeostasis [49, 50]. Increase in plant height, yield components and photosynthetic capacity was found in mung bean genotypes supplemented with bioregulators like salicylic acid and glutathione [51].

TU has the ability to maintain broad range of ROS scavenging within plant cell by altering the intrinsic metabolic events and signaling molecule [50]. In the present study, activities of all the primary antioxidant enzymes and GSH-redox state was marginally varied in As + 6.5 mM TU treated leaves of mung bean seedlings (Table 1), suggesting significant improvement of antioxidant defense capacities. Remarkably enough, APX, DHAR and GR activities were enhanced at 13 mM TU + As treated seedlings and GSH-redox state was hovered around 0.8 (Table 1). This stimulated defense capabilities under the influence of TU effectively scavenged As-generated ROS and effectively countered oxidative damage due to membrane lipid peroxidations, as suggested by quite normal (close to control) level of both H<sub>2</sub>O<sub>2</sub> and MDA in mung bean leaves. A reversal of As-induced oxidative stress through reduction in lipid peroxidation and H<sub>2</sub>O<sub>2</sub> level and stimulation in antioxidant defense machinery was also observed in germinating seeds of mung bean by applying nitric oxide as protective agents and in hydroponically grown mung bean seedlings supplemented with selenium [52, 53].

**TABLE 1** Growth traits and leaf biochemical parameters in mung bean [*Vigna radiata* (L.) Wilczek.] genotype PUSA Baisakhi subjected to 25  $\mu$ M sodium arsenate (As), As + 6.5 mM thiourea (TU) and As + 13 mM TU treatments

Traits	Control	As	As + 6.5 mM TU	As + 13 mM TU
Shoot dry weight plant <sup>-1</sup> (g)	0.17 ± 0.08b	0.08 ± 0.02c	0.15 ± 0.04b	0.23 ± 0.09a
Root dry weight plant <sup>-1</sup> (g)	0.19 ± 0.08b	0.05 ± 0.02c	0.18 ± 0.04b	0.28 ± 0.04a
Chlorophyll a (mg g <sup>-1</sup> FW)	2.98 ± 0.12a	2.08 ± 0.10b	2.93 ± 0.11a	3.03 ± 0.13a
Chlorophyll b (mg g <sup>-1</sup> FW)	1.32 ± 0.07a	1.33 ± 0.10a	1.33 ± 0.11a	1.34 ± 0.13a
Chlorophyll a/b ratio	2.25 ± 1.9a	1.56 ± 1.6b	2.22 ± 2.1a	2.27 ± 2.2a
Carotenoids (mg g <sup>-1</sup> FW)	1.58 ± 0.11a	1.03 ± 0.07c	1.49 ± 0.11b	1.61 ± 0.11a
GSH (nmol g <sup>-1</sup> DW)	33.9 ± 1.7b	17.8 ± 1.1c	30.9 ± 1.5b	40.9 ± 1.6a
GSSG (nmol g <sup>-1</sup> DW)	10.2 ± 0.9b	15.9 ± 1.0a	11.4 ± 1.1b	8.0 ± 1.1b
GSH redox [GSH/GSH+GSSG]	0.770 ± 1.3	0.531 ± 1.3	0.731 ± 1.8	0.841 ± 1.8
SOD (Unit min <sup>-1</sup> mg <sup>-1</sup> protein)	103.8 ± 3.8c	310.1 ± 4.7a	110.6 ± 3.3b	105.3 ± 2.9c
APX (nmol AsA oxidized min <sup>-1</sup> mg <sup>-1</sup> protein)	134.7 ± 4.8b	33.4 ± 1.1c	135.1 ± 4.7b	156.8 ± 5.1a
DHAR ( $\mu$ mol AsA formed min <sup>-1</sup> mg <sup>-1</sup> protein)	1.53 ± 0.6b	0.49 ± 0.09c	1.47 ± 0.7b	1.67 ± 0.9a
GR (nmol NADPH oxi min <sup>-1</sup> mg <sup>-1</sup> protein)	30.3 ± 2.8a	15.5 ± 1.1c	23.7 ± 1.8b	33.8 ± 3.1a
CAT (nmol H <sub>2</sub> O <sub>2</sub> degraded min <sup>-1</sup> mg <sup>-1</sup> protein)	29.7 ± 4.6b	28.8 ± 4.4c	28.3 ± 4.6c	29.8 ± 4.7a
H <sub>2</sub> O <sub>2</sub> ( $\mu$ mol g <sup>-1</sup> FW)	3.3 ± 0.9c	12.8 ± 1.3a	4.6 ± 0.9b	3.5 ± 0.8c
MDA (nmol g <sup>-1</sup> FW)	2.9 ± 0.8c	10.7 ± 0.9a	3.7 ± 0.9b	3.1 ± 0.9c

Data presented here are means  $\pm$  standard error. Different small letters followed by means indicates significant differences at  $P < 0.05$ . DW-dry weight, FW-fresh weight, GSH-reduced glutathione, GSSG-glutathione disulfide, SOD-superoxide dismutase, APX-ascorbate peroxidase, DHAR-dehydroascorbate reductase, GR-glutathione reductase, CAT-catalases, MDA-malondealdehyde

In the present study, powered by favorable redox balance of GSH and elevated antioxidant defense, the plant growth traits and photosynthesis were maintained as normal despite the exposure of seedlings to As. The importance of GSH redox rather than total GSH content have also been observed in the hardy legume *Lathyrus sativus* L. during leaf rolling and stem fasciation events and confirmed the central role of GSH as a down-stream thiol metabolites during cellular events [44, 54]. The results suggested that As can induce oxidative stress in mung bean seedlings,

and that application of TU is effective not only to counter the As-induced oxidative damage but also to improve the plant growth traits and antioxidant defense capabilities even over control levels.

### CONCLUSION

For the first time, effectiveness of TU has been tested in mung bean seedling exposed to moderate concentrations of As. As induced oxidative stress in mung bean leaves but the effect was effectively countered by TU application. TU has also improved the performance of genotype even in the presence of As.

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