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Exogenous thiourea modulates antioxidant defense and glyoxalase systems in lentil genotypes under arsenic stress

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ABSTRACT

Arsenic (As) is a wide-spread toxic and carcinogenic metalloid, affecting crop productivity worldwide. Lentil, an edible grain legume, is increasingly exposed to soil As contamination. However, our understandings regarding mechanistic details and mitigation strategies against As toxicity in edible legume are extremely poor. The main purpose of this study was to investigate the As-effects and its mitigation by thiourea (TU), a sulfhydryl bioregulator, in lentil. Four widely grown lentil genotypes were grown in nutrient media, supplemented with 30 μM sodium As, As + 6.5 mM TU, and As + 13 mM TU, keeping an untreated control for 10 d. As severely affected plant dry weight by accumulating in shoots and roots. However, TU application sequestered As in crop roots and prevented upward translocation of As. TU coordinately modulated glyoxalase (Gly) system I and II (Gly I and II) and ascorbate (AsA)-glutathione (GSH) redox, and antioxidant defense enzymes in both leaves and roots of four genotypes. Elevation of Gly system prevented toxic methylglyoxal overaccumulation, whereas stimulated AsA-GSH cycle enzymes and GSH-s-transferase and catalase effectively scavenged H_2O_2 and prevented reactive oxygen species (ROS)-mediated onset of oxidative damage in four genotypes, as was evident from the ROS-imaging study. Results suggested exogenous TU stimulated the Gly and antioxidant defense in fine tune against As-induced oxidative damage in lentil genotypes.

KEY WORDS: Antioxidant defense, arsenate, glyoxalase system, lentil, thiourea

INTRODUCTION

Better understanding of mechanistic details of plants' response to environmental toxicants can pave the way to develop safe crop in future. Arsenic (As) is a wide-spread toxic and carcinogenic metalloid. As can induce growth inhibition, low productivity, and poor grain quality by inducing oxidative stress in crop plants (Gunes *et al*., 2009; Tripathi *et al*., 2012). Plant experiences oxidative imbalance due to excess generation of reactive oxygen species (ROS) and loss of delicate balance of ROS homeostasis (Finnegan and Chen, 2012). Grain legumes are highly sensitive to As toxicity and being grown in aerobic fields are generally exposed to arsenate (As V) form of As species (Gunes *et al*., 2009). At the cellular level, As V interferes with normal enzymatic functions and disrupts plant growth and metabolisms (Finnegan and Chen, 2012).

Integration among different defense circuits is pivotal during As-tolerance of crop plants. Ascorbate (AsA) and GSH are the two key players in non-enzymatic defense components, and their redox states are more important than their total amount in cell (Foyer and Noctor, 2011; Noctor *et al*., 2012). GSH as a thiol buffer interacts with numerous cellular components and maintains redox homeostasis favorable to reducing environment. The AsA peroxidase (APX), dehydroascorbate (DHA) reductase (DHAR) and glutathione (GSH) reductase (GR) within AsA-GSH cycle and catalases (CAT) and GSH-s-transferase (GST) outside this cycle are the predominant enzymatic antioxidant defense components against ROS-induced oxidative imbalance in cell (Foyer and Noctor, 2003; 2011; Noctor *et al*., 2012). Besides GSH and GSH-dependent antioxidant defense, methylglyoxal (MG) detoxification systems powered by glyoxalase (Gly) I and II enzymes play significant roles in drought and salinity stress and heavy metal detoxification system in plants (Yadav *et al*., 2005; Singla-Pareek *et al*., 2008; Hossain and Fujita, 2010; Hossain *et al*., 2010). MG is highly toxic to plant cells and by reacting with

proteins, lipids, carbohydrates, and DNA; they can lead to cell death in the absence of any effective protection. In plants, MG is detoxified mainly via the Gly system that is comprised two enzymes: While Gly I converts MG to S-D-lactoylglutathione (SLG) by utilizing GSH, Gly II converts SLG to D-lactic acid, and during this reaction GSH is regenerated. Overexpressions of the Gly pathway in transgenic tobacco and rice plants experiencing environmental stresses can prevent ROS and MG accumulation by maintaining GSH redox homeostasis and Gly activity levels (Yadav *et al.*, 2005; Singla-Pareek *et al.*, 2008).

Priming of existing defense mechanisms without any genome modification has been found effective during crops' tolerance to stresses (Sahu and Singh 1995; Srivastava *et al.*, 2011). Use of sulfhydryl bio-regulator like thiourea (TU), an ROS-scavenger, has been found highly effective in ameliorating salt, ultraviolet, and heat stress in cereals and oilseeds (Srivastava *et al.*, 2011; Akladious, 2014) and in regulating source-to-sink relationship in Indian mustard through alterations in antioxidant defense (Pandey *et al.*, 2013). Among edible legumes, exogenous TU was found to be primarily effective against As-induced oxidative stress in mung bean (Talukdar, 2014), but no such information is available in lentil crop which is extensively grown as grain legume in Indian sub-continent (IIPR, 2011). Primary reports indicate that As exposure can inhibit plant growth and seed yield in lentil genotypes (Talukdar, 2013b; Talukdar and Talukdar, 2014). However, nothing is known regarding mechanistic details of Gly systems and its interaction with GSH-dependent antioxidant defense in any of the grain legume crops under As stress.

Present work was, therefore, designed to: (i) Unravel the response of antioxidant defense and (ii) reveal the modulation of Gly systems under As alone and As + TU applications. The study for the first time indicates coordinated responses between primary antioxidant defense components and MG-detoxification systems during As exposure and their modulations during TU-mediated amelioration of As-stress in lentil genotypes. Apart from physiological and biochemical studies, this fact has been confirmed by ROS imaging analysis in this study.

MATERIALS AND METHODS

Plant Materials and Treatment Protocols

Fresh and healthy seeds of four lentil genotypes (*Lens culinaris* Medik. cv. L 9, Pusa 4, K 75, and PL 234) were surface-sterilized with NaOCl (0.1%, w/v), washed under running tap water followed by distilled water, and were allowed to germinate in the dark in three separate sets on moistened filter paper at 25°C. Germinated seedlings were immediately placed in polythene pots (10 plants/pots) containing 300 ml of Hoagland's No. 2 nutrient media following earlier protocol (Talukdar, 2013a), and were allowed to grow for 10 days. The plants were, then, subjected to the following treatment protocols as: (a) Untreated control, (b) 30 µM sodium As (As, MW 312.01 g/mol; technical grade, purity 98.5%, Sigma-Aldrich), (c) 30 µM As + 6.5 mM TU, and (d) 30 µM As + 13 mM TU. Each treatment was replicated four times. TU (Sigma-Aldrich, Bengaluru, India), a sulfhydryl bio-regulator, was used to pre-soak the seeds in the last two protocols. Pilot experiments were carried out to determine the effective concentrations of TU and As. Control and treated plants were allowed to grow for another 10 days. Nutrient solution was refreshed in every alternate day to prevent depletion of nutrients, TU as well as As in the course of the plant's exposure to them. The experiment was carried out in a completely randomized block design in a controlled growth chamber under a 14 h photoperiod, 26/16 (±2°C), relative humidity of 72 ± 2% and a photosynthesis photon flux density of 180 µmol m^{-2} s^{-1}. Plants were harvested after a stipulated period. Plant parts were separated and thoroughly washed and oven dried at 72°C for 48 h to measure dry weights of shoots and roots.

Determination of Chlorophyll (Chl) and Total Carotenoids

Leaf Chl and carotenoid contents were determined (Lichtenthaler, 1987). Leaf tissue (50 mg) was homogenized in 10 ml chilled acetone (80%). The homogenate was centrifuged at 4000 *g* for 12 min. The absorbance of the supernatant was recorded at 663, 647, and 470 nm for Chl A, Chl B, and carotenoids, respectively. The contents were expressed as mg Chl or carotenoids g^{-1} fresh weight (FW).

Measurement of As Content

As concentration in dried shoot and root samples was measured by digestion methods (HNO_3-$HClO_4$ mixture at 3:1, v/v) using flow injection-hydride generation atomic absorption spectrophotometer (Perkin-Elmer, FIA-HAAS Analyst 400) and keeping Standard Reference Materials of tomato leaves (item number 1573a, from National Institute of Standards and Technology, USA) as part of the quality assurance/quality control protocol (Talukdar, 2013a). The translocation factor (TF) is the ratio of the level of As in shoots on roots.

Determination of GSH, Ascorbate Content, and Assay of Antioxidant Defense Enzymes

Reduced and oxidized form of AsA and GSH were determined according to Law *et al*. (1983) and Griffith (1980), respectively. For enzyme assay, plant tissue of 250 mg was ground in liquid nitrogen and homogenized in 1 ml of 50 mM K-phosphate buffer (pH 7.8) containing 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), and 2% (w/v) polyvinyl pyrrolidone using a chilled mortar and pestle kept in an ice bath. The homogenate was centrifuged at 15,000 g at 4°C for 20 min. Clear supernatant was used for enzyme assays. For measuring APX activity, the tissue was separately grounded in homogenizing medium containing 2.0 mM AsA in addition to the other ingredients. All assays were done at 0-4°C. Soluble protein content was determined using BSA as a standard (Bradford, 1976).

Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured by nitro blue tetrazolium (NBT) photochemical assay (Beyer and Fridovich, 1987). One unit of SOD was equal to that amount causing a 50% decrease of SOD-inhibited NBT reduction. APX (EC 1.11.1.11) activity (µmol AsA oxidized/min/mg protein) was assayed following the protocol of Nakano and Asada (1981). Three ml of the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 0.5 mM AsA, 0.1 mM H_2O_2, and 0.1 ml enzyme extract. The H_2O_2-dependent oxidation of AsA was followed by a decrease in the absorbance at 290 nm ($\varepsilon = 2.8$/mM/cm). APX activity was expressed as nmol AsA oxidized/min/mg protein. For DHAR (EC 1.8.5.1) activity assay, the reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.2 mM DHA, 2.5 mM GSH, and 0.1 mM EDTA in a final volume of 1 ml. Reaction was started by addition of suitable aliquots of enzyme extract. The increase in absorbance was recorded at 30 s intervals for 3 min at 265 nm. Enzyme activity was expressed as µmol AsA formed/min/mg protein (Nakano and Asada, 1981). For GR (EC 1.6.4.2) assay, enzyme activity was determined by monitoring the GSH-dependent oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) (Carlberg and Mannervik, 1985). In a cuvette, 0.75 ml 0.2 M potassium phosphate buffer (pH 7.0) containing 75 µl NADPH (2 mM), 2 mM EDTA, and 75 µl oxidized GSH (20 mM) were mixed. 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/min/mg protein. CAT (EC 1.11.1.6) extraction was performed in a 50 mM Tris-HCl buffer. Activity was assayed by measuring the reduction of H_2O_2 at 240 nm ($\varepsilon = 39.4$/mM/cm) and 25°C as detailed earlier (Talukdar, 2013a; 2013b). For estimation of GSTs (EC 2.5.1.18) specific activity, 1 g of plant samples was extracted in 5 ml medium containing 50 mM phosphate buffer, pH 7.5, 1 mM DTT, and 1 mM EDTA. The reaction mixture contained 50 mM phosphate buffer, pH 7.5, 1 mM 1-chloro-2,4-dinitrobenzene, and the elute equivalent to 100 µg of protein. The reaction was initiated with the addition of 1 mM GSH, and formation of S-(2,4-dinitrophenyl) GSH (DNP-GS) was monitored as an increase in absorbance at 334 nm to calculate the GST specific activity (Li *et al*., 1995).

Gly I (EC: 4.4.1.5) assay was carried out according to Hossain and Fujita (2010). Briefly, the assay mixture contained 100 mM K-phosphate buffer (pH 7.0), 15 mM magnesium sulfate, 1.7 mM GSH, and 3.5 mM MG in a final volume of 0.7 ml. The reaction was started by the addition of MG, and the activity was calculated at 240 nm for 1 min ($\varepsilon = 3.37$/mM/cm).

Gly II (EC: 3.1.2.6) activity was determined following Principato *et al*. (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB, and 1 mM SLG in a final volume of 1 ml. The reaction was started by the addition of SLG, and the activity was calculated ($\varepsilon = 13.6$/mM/cm).

Determination of MG Level

Plant tissues were homogenized in 5% perchloric acid and centrifuged at 4°C for 10 min at 11,000 *g*. The supernatant was decolorized by adding charcoal; then, centrifuged at 11,000 *g* for 12 min. The supernatant neutralized by a saturated solution of potassium carbonate at room temperature was used for MG estimation by adding sodium dihydrogen phosphate and 20 µl of freshly prepared 0.5 M *N*-acetyl-L-cysteine to a final volume of 1 ml. Formation of the product *N*-α-acetyl-*S*-(1-hydroxy-2-oxo-prop-1-yl) cysteine was recorded after 10 min at 288 nm (Wild *et al*., 2012). The MG content was calculated by the standard curve and expressed as µmol/g/FW.

Determination of H_2O_2 Content, Membrane Lipid Peroxidation, and Electrolyte Leakage (EL)

H_2O_2 was estimated following Wang *et al*. (2007) from the absorbance at 410 nm using a standard curve. Lipid peroxidation rates were determined by measuring the malondialdehyde (MDA) equivalents according to Hodges *et al*. (1999). EL% was measured according to Dionisio-Sese and Tobita (1998).

ROS Imaging

Detection and imaging of superoxide radicals in leaf and root sections were carried out using the fluorescence probe dihydroethidium (DHE) (Rodr′ıguez-Serrano *et al.*, 2006). Leaf/root segments of approximately 30 mm^2 were incubated for 1 h at 25°C, in darkness, with 10 μM DHE prepared in 5 mM Tris-HCl buffer at pH 7.4, and samples were washed twice with the same buffer for 12 min each. After washing, sections were embedded in a mixture of 15% acrylamide-bisacrylamide stock solution, and 100 mm thick sections, were cut under 10 mM phosphate-buffered saline (PBS). Sections were then soaked in glycerol: PBS (containing azide) (1:1 v/v) and mounted in the same medium for examination with a confocal LASER scanning microscopy (CLSM) system (Carl Zeiss, LSM 780, Bengaluru, India) using standard filters and collection modalities for DHE green fluorescence (λ excitation 488 nm; λ emission 520 nm). H_2O_2 was detected by incubation with 25 μM 2'7'-dichloro fluorescein diacetate (DCF-DA) (excitation 485 nm, emission 530 nm) (Rodr′ıguez-Serrano *et al.*, 2006). Preinfused sections with 1 mM tetramethylpiperidinyloxy (TMP), a scavenger of superoxide radicals, and 1 mM AsA, a scavenger of H_2O_2 served as negative controls.

Statistical Analysis

The results are the mean values ± standard errors of at least four replicates. Multiple comparisons of means were performed by ANOVA (SPSS Inc., version 10), and the means were separated by Duncan's multiple range test with significance level at $P < 0.05$. Simple correlation was carried out among different traits using Microsoft Excel data analysis tool pack 2007.

RESULTS

Changes in Plant Height, Dry Weight, and As Uptake Potential

Lentil genotypes exhibited significant growth inhibition as both root and shoot length (SL) and dry weights reduced significantly ($P < 0.05$) in comparison to control at 30 μM As. Stem and root length (RL) in lentil genotypes were reduced by 2-2.5-fold (Table 1). In L 9 and Pusa 4, while shoot dry weight (SDW) was reduced by about 2-2.7-fold root dry weight (RDW) was decreased by about 3-3.3-fold (Table 1). SDW in K 75 and PL 234 were declined by nearly 1.8-fold and 3.1-fold, respectively, whereas RDW was decreased by about 2.5-fold and 4.5-fold, respectively. Co-application of 6.5 mM TU with As did not change SL and RL as well as SDW and RDW significantly in L 9 and Pusa 4. However, both dry weights increased over control by about 1.5-fold at As + 13 mM TU in both the genotypes (Table 1). SDW and RDW exhibited upward trend in K 75 and Pl 234 genotypes at As + 6.5 mM TU and further increased at As + 13 mM TU (Table 1). At 30 μM, As accumulated in marginally higher amount in roots of all four genotypes than that of shoots (shoot/root TF = 1.0). Application of TU at 6.5 mM and 13 mM significantly increased As amount in roots compared to shoots (shoots/roots TF <1.0) of all four genotypes but both the K 75 and PL 234 accumulated significantly higher As content than L 9 and Pusa 4 in their roots in presence of TU (Figure 1a).

Changes in Leaf Photosynthetic Pigment

Leaf Chl A, Chl A/B ratio, and carotenoids reduced significantly in four genotypes with different magnitudes

Table 1: Changes in RL (cm/plant), SL (cm/plant), SDW (g/plant), RDW (g/plant) in L 9, Pusa 4, K 75, and PL 234 genotypes of lentils under untreated control, sodium arsenate (30 μM As), As+6.5 mM TU and As+13 mM TU for 10 days treatment duration

Treatments (traits)	L 9	Pusa 4	K 75	PL 234
Control (RL)	$28.88\pm1.8^{bb'}$	$20.89\pm1.3^{cb'}$	$33.45\pm2.1^{aa'}$	$36.87\pm1.9^{aa'}$
As (RL)	$11.56\pm1.1^{bb'}$	$8.41\pm1.0^{bb'}$	$16.72\pm1.3^{aa'}$	$16.76\pm1.3^{aa'}$
As+6.5 mM TU (RL)	$29.03\pm1.9^{bb'}$	$21.03\pm1.4^{cb'}$	$33.57\pm2.3^{aa'}$	$37.00\pm2.9^{aa'}$
As+13 mM TU (RL)	$33.03\pm2.6^{ba'}$	$28.08\pm1.6^{cb'}$	$36.51\pm2.8^{aa'}$	$37.88\pm3.2^{aa'}$
Control (SL)	$31.45\pm1.9^{bb'}$	$27.56\pm1.2^{bb'}$	$39.11\pm2.0^{aa'}$	$37.22\pm1.9^{aa'}$
As (SL)	$12.60\pm1.4^{bb'}$	$11.02\pm1.0^{bb'}$	$20.08\pm1.4^{aa'}$	$17.01\pm1.5^{aa'}$
As+6.5 mM TU (SL)	$31.99\pm2.0^{bb'}$	$28.11\pm1.2^{bb'}$	$40.12\pm2.0^{aa'}$	$37.76\pm1.9^{aa'}$
As+13 mM TU (SL)	$32.11\pm2.2^{bb'}$	$29.13\pm1.3^{bb'}$	$44.32\pm2.9^{aa'}$	$39.52\pm2.3^{aa'}$
Control (SDW)	$0.19\pm0.04^{bb'}$	$0.21\pm0.04^{bb'}$	$0.30\pm0.06^{aa'}$	$0.32\pm0.08^{aa'}$
As (SDW)	$0.10\pm0.02^{bb'}$	$0.08\pm0.01^{bb'}$	$0.17\pm0.03^{aa'}$	$0.19\pm0.02^{aa'}$
As+6.5 mM TU (SDW)	$0.20\pm0.04^{bb'}$	$0.22\pm0.04^{bb'}$	$0.31\pm0.07^{aa'}$	$0.34\pm0.09^{aa'}$
As+13 mM TU (SDW)	$0.29\pm0.09^{bb'}$	$0.32\pm0.10^{bb'}$	$0.43\pm0.12^{aa'}$	$0.46\pm0.17^{aa'}$
Control (RDW)	$0.24\pm0.03^{bb'}$	$0.26\pm0.04^{bb'}$	$0.36\pm0.09^{aa'}$	$0.39\pm0.10^{aa'}$
As (RDW)	$0.08\pm0.01^{bb'}$	$0.08\pm0.01^{bb'}$	$0.14\pm0.02^{aa'}$	$0.15\pm0.01^{aa'}$
As+6.5 mM TU (RDW)	$0.23\pm0.03^{bb'}$	$0.27\pm0.04^{bb'}$	$0.37\pm0.09^{aa'}$	$0.41\pm0.11^{aa'}$
As+13 mM TU (RDW)	$0.36\pm0.10^{bb'}$	$0.39\pm0.10^{bb'}$	$0.47\pm0.14^{aa'}$	$0.50\pm0.16^{aa'}$

Means±SE of four replicates treatment^{-1}. Means followed by different lowercase letters in rows (genotypes) and prime lowercase letters in columns (treatments) are significantly different at $P<0.05$ by ANOVA followed by Duncan's multiple range test. RL: Root length, SL: Shoot length, SDW: Shoot dry weight, RDW: Root dry weight, TU: Thiourea, As: Arsenic

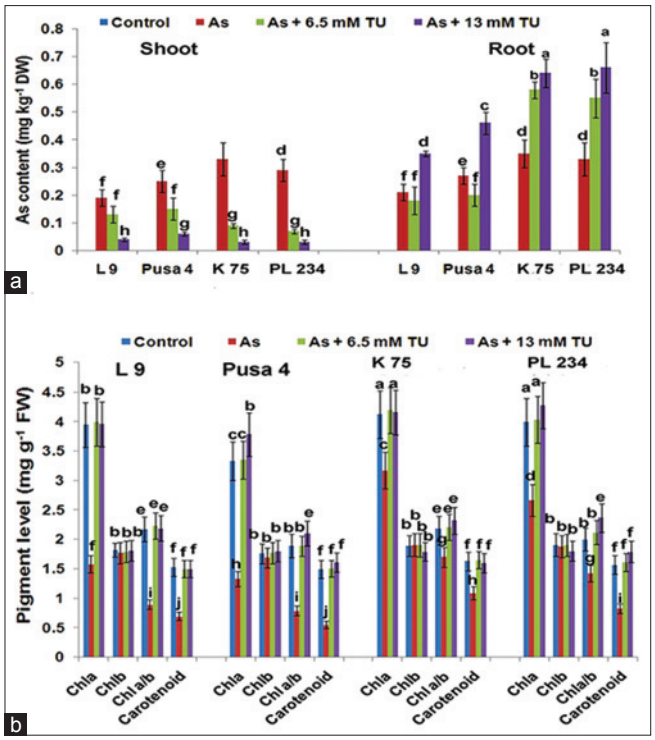

Figure 1: Changes in (a) Arsenic (As) content in shoots and roots and (b) leaf chlorophyll (Chl) A, B, Chl A/B ratio, and carotenoid contents of four lentil genotypes (L 9, Pusa 4, K 75, and PL 234) under untreated control, 30 µM arsenate and As + thiourea (TU) treatments. Data are means ± standard error of four replicates with different lowercase letters over error bars represent significant differences ($P < 0.05$) at ANOVA followed by Duncan's multiple range test

(Figure 1b). As treatment alone reduced CHL A content by about 2.5-3.2-fold in L 9 and Pusa 4 and 1.3-1.5-fold in K 75 and PL 234 genotypes. CHL B content did not change significantly, but Chl A/B ratio reduced markedly in all genotypes. The carotenoid level was also severely affected with nearly 2.2 (L 9) to 2.7-fold (Pusa 4) decrease and 1.5 (K 75) to 1.9 (PL 234)-fold reduction in lentil genotypes (Table 1). Application of As + 6.5 mM TU and As + 13 mM TU considerably restored the photosynthetic pigment levels in four genotypes. Chl A content, Chl A/B ratio, and carotenoid levels even increased significantly in Pusa 4 and PL 234 exposed to As + 13 mM TU (Figure 1b). Chl A/B ratio was significantly correlated with SDW in L 9 ($r = 0.703$, $n = 12$, $P < 0.05$), Pusa 4 ($r = 0.710$, $n = 12$, $P < 0.05$), K 75 ($r = 0.813$, $n = 12$, $P < 0.05$), and PL 234 ($r = 0.789$, $n = 12$, $P < 0.05$) genotypes (data not in table).

Response of Antioxidant Defense Components and Gly System

Foliar and roots AsA and GSH content decreased while DHA and GSH disulfide (GSSG) level increased significantly over control during As exposure alone (Table 2). Among the four genotypes, As-treatment reduced AsA and GSH levels in both organs of L 9 and Pusa 4 in higher extent than that in K 75 and PL 234 (Table 2). Compared to control, AsA and GSH redox values decreased in all four genotypes, but foliar AsA and GSH redox did not change significantly among the genotypes. Respective redox values in roots, however, were significantly higher in K 75 and PL 234 (Table 2). At As + 6.5 mM TU, AsA level was remained low in L 9 and Pusa 4 but comparable to control in K 75 and PL 234. GSH and GSSG content were comparable to control in all four genotypes (Table 2). Upon exposed to As + 13 mM TU, root AsA, and GSH content significantly increased, but DHA and GSSG levels decreased in the genotypes. Leaf AsA and GSH did not change significantly in the four genotypes compared to their respective controls. AsA and GSH redox changed, accordingly (Table 2).

Antioxidant enzymatic activities also differed sharply between presence and absence of TU. As exposure alone reduced foliar and root APX, DHAR, GR, and GSTs activities in all four genotypes but the treatment enhanced SOD activity (Figure 2a-e). CAT activity did not change significantly in K 75 and PL 234 but declined significantly in L 9 and Pusa 4 seedlings (Figure 2f). Upon imposition of As + 6.5 mM TU, foliar and root activities of APX, DHAR, GR, and GSTs became as per control in all genotypes, but CAT level was remained low. At As + 13 mM TU, activities of five enzymes were significantly higher in roots but were comparable in leaves of all four genotypes (Figure 2a-c, e, f). SOD activity, however, was noticeably low at As + 6.5 mM TU, and further reduced at As + 13 mM TU (Figure 2d).

As treatment significantly reduced Gly I and II activities in leaves and roots of L 9 and Pusa 4 genotypes (Figure 3a and b). Gly I activity did not change significantly, but Gly II level decreased markedly in As-treated K 75 and PL 234 (Figure 3a and b). At As + 6.5 mM TU, Gly I and II activities became comparable to control in leaves of four genotypes but increased markedly in their roots. At As + 13 mM TU, root Gly I and II activity increased significantly in the genotypes, but the foliar activity of both enzymes did not change significantly ($P > 0.05$) in all four genotypes under As + 13 mM TU (Figure 3a and b).

Changes in Methylglyoxal, H_2O_2, and Oxidative Stress Level

As treatment alone significantly elevated MG and other oxidative stress markers such as H_2O_2, MDA, and EL% in leaves and roots of four genotypes (Table 3). MG level

Table 2: Changes in AsA (μmol/g FW), DHA (μmol/g FW), glutathione (GSH, nmol/g FW), and GSSG (nmol/g FW) content in leaves and roots of L 9, Pusa 4, K 75, and PL 234 genotypes of lentils under untreated control, sodium As (30 μM As), As+6.5 mM (TU) and As+13 mM TU for 10 d treatment duration

Traits	Treatments	L 9	Pusa 4	K 75	PL 234
AsA (leaf)	Control	$1.09\pm0.37^{aa'}$	$0.98\pm0.13^{ab'}$	$1.27\pm0.34^{aa'}$	$1.34\pm0.26^{aa'}$
	As	$0.54\pm0.07^{bb'}$	$0.49\pm0.06^{bb'}$	$0.77\pm0.07^{ba'}$	$0.80\pm0.08^{ba'}$
	As+6.5 mM TU	$0.58\pm0.08^{bb'}$	$0.51\pm0.07^{bb'}$	$1.28\pm0.38^{aa'}$	$1.29\pm0.18^{aa'}$
	As+13 mM TU	$1.10\pm0.38^{ab'}$	$1.00\pm0.15^{ab'}$	$1.31\pm0.39^{aa'}$	$1.32\pm0.28^{aa'}$
AsA (root)	Control	$0.99\pm0.28^{ba'}$	$0.97\pm0.16^{ba'}$	$1.03\pm0.19^{ba'}$	$1.05\pm0.21^{ba'}$
	As	$0.50\pm0.06^{cb'}$	$0.51\pm0.05^{cb'}$	$0.71\pm0.11^{ca'}$	$0.69\pm0.10^{ca'}$
	As+6.5 mM TU	$1.01\pm0.29^{ba'}$	$0.97\pm0.18^{ba'}$	$1.05\pm0.31^{ba'}$	$1.07\pm0.34^{ba'}$
	As+13 mM TU	$1.87\pm0.33^{ab'}$	$1.85\pm0.34^{ab'}$	$1.99\pm0.45^{aa'}$	$1.98\pm0.39^{aa'}$
DHA (leaf)	Control	$0.12\pm0.03^{ba'}$	$0.14\pm0.02^{ba'}$	$0.14\pm0.02^{ba'}$	$0.15\pm0.03^{ba'}$
	As	$0.39\pm0.05^{ac'}$	$0.60\pm0.06^{aa'}$	$0.45\pm0.07^{ac'}$	$0.53\pm0.09^{ab'}$
	As+6.5 mM TU	$0.17\pm0.02^{ab'}$	$0.17\pm0.07^{ab'}$	$0.22\pm0.07^{aa'}$	$0.27\pm0.08^{aa'}$
	As+13 mM TU	$0.09\pm0.02^{ba'}$	$0.11\pm0.04^{ca'}$	$0.10\pm0.03^{ca'}$	$0.13\pm0.02^{ba'}$
DHA (root)	Control	$0.11\pm0.02^{ba'}$	$0.11\pm0.03^{ca'}$	$0.12\pm0.02^{ba'}$	$0.12\pm0.04^{ba'}$
	As	$0.60\pm0.08^{aa'}$	$0.68\pm0.09^{aa'}$	$0.48\pm0.03^{ab'}$	$0.47\pm0.03^{ab'}$
	As+6.5 mM TU	$0.16\pm0.06^{aa'}$	$0.15\pm0.05^{ba'}$	$0.13\pm0.03^{ba'}$	$0.15\pm0.05^{aa'}$
	As+13 mM TU	$0.10\pm0.02^{ba'}$	$0.09\pm0.01^{ca'}$	$0.10\pm0.02^{ba'}$	$0.11\pm0.01^{ba'}$
AsA redox (leaf)	Control	$0.901\pm0.04^{ba'}$	$0.876\pm0.05^{bb'}$	$0.900\pm0.05^{aa'}$	$0.899\pm0.05^{aa'}$
	As	$0.580\pm0.06^{db'}$	$0.450\pm0.02^{cc'}$	$0.670\pm0.03^{ca'}$	$0.601\pm0.02^{cb'}$
	As+6.5 mM TU	$0.773\pm0.06^{cb'}$	$0.750\pm0.04^{cb'}$	$0.853\pm0.09^{ba'}$	$0.830\pm0.08^{ba'}$
	As+13 mM TU	$0.924\pm0.09^{aa'}$	$0.900\pm0.08^{aa'}$	$0.930\pm0.09^{aa'}$	$0.910\pm0.09^{aa'}$
AsA redox (root)	Control	$0.900\pm0.05^{aa'}$	$0.899\pm0.05^{aa'}$	$0.896\pm0.05^{ba'}$	$0.897\pm0.04^{ba'}$
	As	$0.455\pm0.05^{cb'}$	$0.429\pm0.07^{cb'}$	$0.597\pm0.08^{ca'}$	$0.595\pm0.08^{ca'}$
	As+6.5 mM TU	$0.863\pm0.08^{ba'}$	$0.867\pm0.08^{ba'}$	$0.890\pm0.09^{ba'}$	$0.877\pm0.08^{ba'}$
	As+13 mM TU	$0.950\pm0.09^{aa'}$	$0.954\pm0.09^{aa'}$	$0.952\pm0.09^{aa'}$	$0.947\pm0.09^{aa'}$
GSH (leaf)	Control	$177.8\pm3.9^{aa'}$	$181.9\pm4.0^{aa'}$	$209.6\pm5.2^{aa'}$	$212.8\pm5.8^{aa'}$
	As	$88.3\pm2.5^{bb'}$	$89.5\pm2.6^{bb'}$	$100.3\pm3.8^{ba'}$	$105.2\pm3.2^{ba'}$
	As+6.5 mM TU	$175.5\pm4.1^{ab'}$	$179.8\pm4.0^{ab'}$	$210.2\pm5.5^{aa'}$	$209.7\pm5.3^{aa'}$
	As+13 mM TU	$170.4\pm3.9^{ab'}$	$180.4\pm3.7^{ab'}$	$205.9\pm4.7^{aa'}$	$210.2\pm5.2^{aa'}$
GSSG (leaf)	Control	$19.8\pm1.8^{ba'}$	$22.7\pm1.6^{ba'}$	$25.3\pm2.3^{ba'}$	$20.9\pm1.9^{ba'}$
	As	$59.9\pm2.6^{aa'}$	$61.8\pm3.1^{aa'}$	$67.7\pm2.8^{aa'}$	$60.3\pm2.2^{aa'}$
	As+6.5 mM TU	$20.1\pm1.6^{ba'}$	$22.5\pm1.5^{ba'}$	$25.6\pm2.2^{ba'}$	$21.0\pm2.0^{ba'}$
	As+13 mM TU	$19.7\pm1.8^{ba'}$	$20.8\pm1.8^{ba'}$	$24.8\pm2.2^{ba'}$	$18.8\pm1.8^{ca'}$
GSH (root)	Control	$109.5\pm1.5^{bc'}$	$170.8\pm1.6^{bb'}$	$198.5\pm1.9^{ba'}$	$189.8\pm1.8^{ba'}$
	As	$70.8\pm2.1^{cb'}$	$70.3\pm2.3^{cb'}$	$106.8\pm1.5^{ca'}$	$103.5\pm1.3^{ca'}$
	As+6.5 mM TU	$110.0\pm1.5^{bc'}$	$171.1\pm1.7^{bb'}$	$200.1\pm2.2^{ba'}$	$190.3\pm1.9^{ba'}$
	As+13 mM TU	177.7 ± 2.4^{a}	190.8 ± 2.6^{a}	223.7 ± 3.1^{a}	228.9 ± 3.1^{a}
GSSG (root)	Control	$17.6\pm1.1^{ba'}$	$20.7\pm1.5^{ba'}$	$18.6\pm1.4^{ba'}$	$22.6\pm2.4^{ba'}$
	As	$79.8\pm2.3^{aa'}$	$72.3\pm2.5^{aa'}$	$78.7\pm2.2^{aa'}$	$84.4\pm3.8^{aa'}$
	As+6.5 mM TU	$17.5\pm1.1^{ba'}$	$21.0\pm2.1^{ba'}$	$18.5\pm1.3^{ba'}$	$23.1\pm2.3^{ba'}$
	As+13 mM TU	$8.78\pm0.12^{ca'}$	$9.11\pm0.15^{ca'}$	$10.0\pm0.10^{ca'}$	$6.18\pm0.67^{cb'}$
GSH redox (leaf)	Control	$0.899\pm0.09^{aa'}$	$0.889\pm0.09^{aa'}$	$0.892\pm0.09^{aa'}$	$0.910\pm0.09^{aa'}$
	As	$0.594\pm0.05^{ba'}$	$0.591\pm0.06^{ba'}$	$0.595\pm0.06^{ba'}$	$0.633\pm0.06^{ba'}$
	As+6.5 mM TU	$0.897\pm0.08^{aa'}$	$0.891\pm0.09^{aa'}$	$0.891\pm0.09^{aa'}$	$0.909\pm0.09^{aa'}$
	As+13 mM TU	$0.896\pm0.08^{aa'}$	$0.900\pm0.09^{aa'}$	$0.892\pm0.08^{aa'}$	$0.917\pm0.09^{aa'}$
GSH redox (root)	Control	$0.861\pm0.08^{ba'}$	$0.891\pm0.08^{ba'}$	$0.914\pm0.09^{ba'}$	$0.893\pm0.08^{aa'}$
	As	$0.470\pm0.05^{cb'}$	$0.493\pm0.05^{cb'}$	$0.575\pm0.06^{ca'}$	$0.551\pm0.06^{ba'}$
	As+6.5 mM TU	$0.863\pm0.08^{bb'}$	$0.891\pm0.09^{ba'}$	$0.915\pm0.09^{ba'}$	$0.892\pm0.08^{aa'}$
	As+13 mM TU	$0.953\pm0.09^{aa'}$	$0.954\pm0.09^{aa'}$	$0.957\pm0.09^{aa'}$	$0.974\pm0.09^{aa'}$

Means±SE of four replicates treatment^{-1}. Means followed by different lowercase letters in columns and lower case letters with prime in rows are significantly different at $P<0.05$ by ANOVA following Duncan's multiple range test. AsA: Ascorbate, DHA: Dehydroascorbate, GSSG: Glutathione disulfide, SE: Standard error, FW: Fresh weight, As: Arsenic, SE: Standard error

reduced significantly in both organs of all four genotypes on imposition of As + 6.5 mM TU and further reduced at As + 13 mM TU (Table 3). Foliar and root H_2O_2, MDA and EL% increased to the tune of 3-6-fold in leaves and roots of the genotypes exposed to As only. During As + TU treatment, H_2O_2 level decreased substantially which was accompanied by normal (comparable to control) to reduced level of MDA and EL% in leaves and roots of all four genotypes (Table 3).

ROS Imaging Study

In leaves and roots of untreated controls, faint red (superoxide) and green (H_2O_2) fluorescence was detected in the vascular region (Figure 4a, b, f, g). However, in As-treated genotypes, bright red and green fluorescence was detected in vascular bundle regions, endodermis, sclerenchyma, mesophyll (leaf), and epidermis (Figure 4c-e, h, i). Pre-incubation of sections with 1 mM TMP (a superoxide

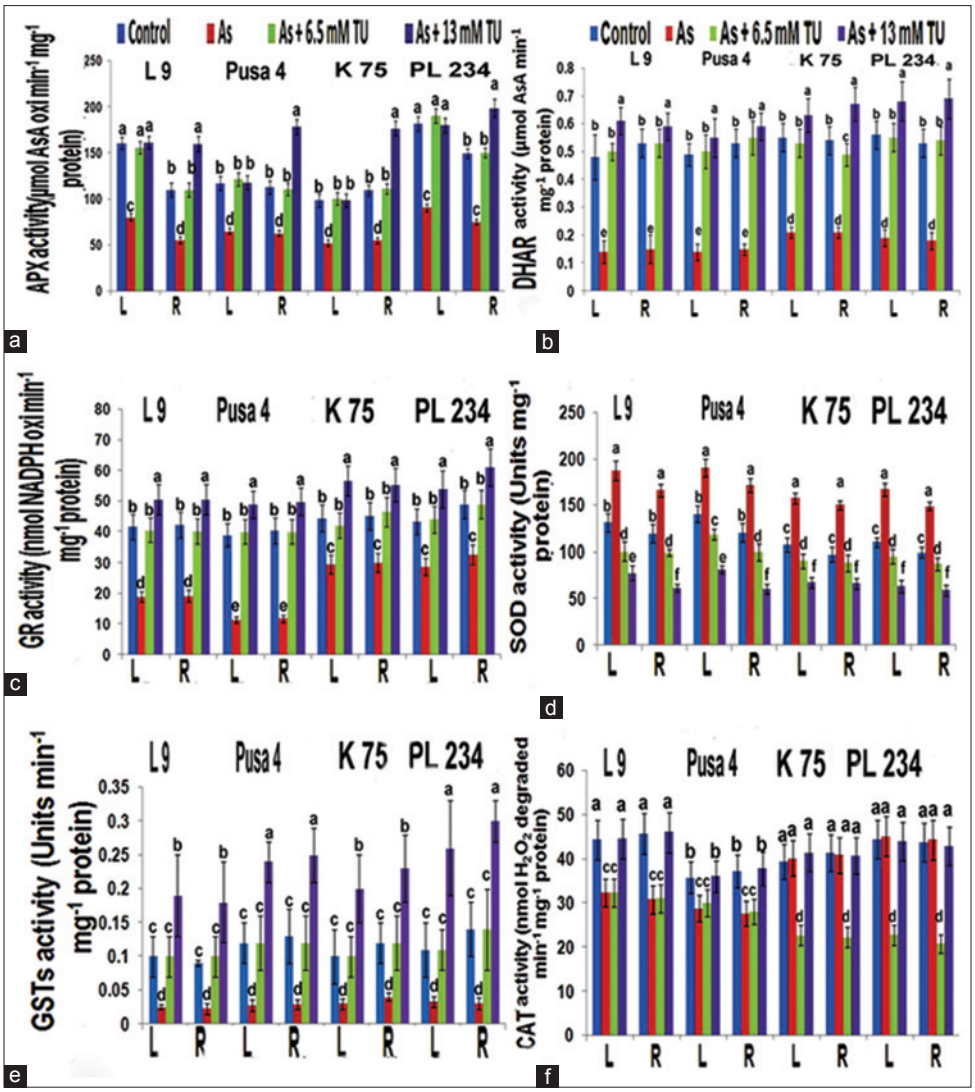

Figure 2: Changes in (a) Ascorbate peroxidase, (b) dehydroascorbate reductase, (c) glutathione (GSH) reductase, (d) superoxide dismutase, (e) GSH-s-transferases, and (f) catalases activities in leaves and roots of four lentil (L 9, Pusa 4, K 75, and PL 234) genotypes under untreated control, 30 μM arsenate and arsenic + thiourea treatments. Data are means ± standard error of four replicates with different lowercase letters over error bars represent significant differences ($P < 0.05$) at ANOVA followed by Duncan's multiple range test

scavenger) and AsA (an H_2O_2 scavenger) resulted in significant reduction of the fluorescence (Figure 4j-l). Upon As + 6.5 mM TU, mild DHE (red) and DCF-DA (green) fluorescence was localized only in vascular bundle and epidermis of four genotypes which was further reduced at 13 mM TU + As (Figure 5a-h).

DISCUSSION

Growth inhibition is one of the significant primary physiological phenomena during As-toxicity of crop plants (Stoeva *et al.*, 2005; Gupta *et al.*, 2008; Malik *et al.*, 2012; Talukdar, 2013b). In this study, reduction in dry weight was mainly due to decrease in stem and RL in four genotypes exposed to As-treatment alone. However, genotypes differed significantly in their responses toward As-treatment. The L 9 and Pusa 4 suffered greater inhibition of SL and RL as well as SDW and RDW than K 75 and PL 234. The present finding was consistent with earlier reports on As-induced growth inhibition of lentil, grass pea, common beans, chickpea, rice, and mustard crops with significant genotypic differences (Chaturvedi, 2006; Gunes *et al.*, 2009; Talukdar, 2013c; 2015; Talukdar and Talukdar, 2014). Here, upon application of TU, significant improvement of plant growth was evident from comparable SDW and RDW at 6.5 mM, and thereafter, their increase over control in L 9 and Pusa 4 at 13 mM. More significantly, in the case of both K 75 and PL 234, SDW and RDW increased over control at 6.5 mM TU and further increased at 13 mM TU. Interestingly, As accumulation pattern in the absence of TU was nearly comparable between roots and shoots

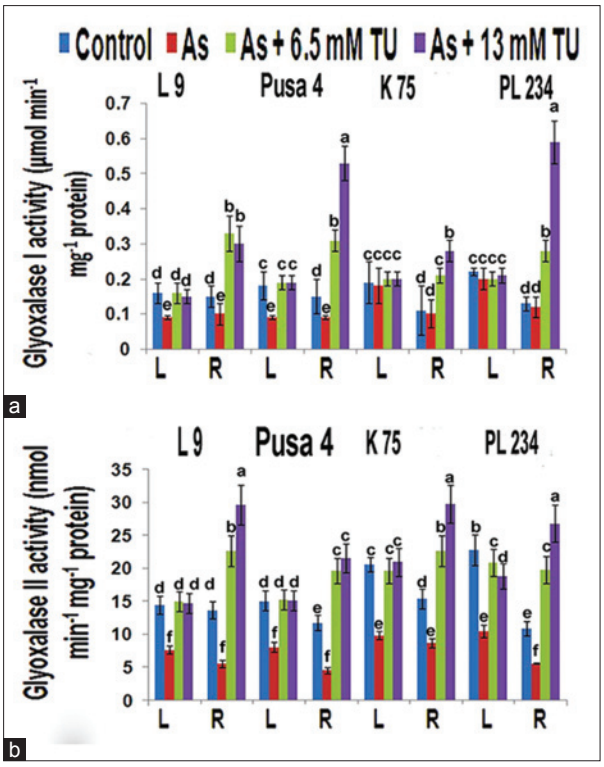

Figure 3: Changes in (a) Glyoxalase (Gly) I and (b) Gly II activities in leaves and roots of two lentils (L 9 and Pusa 4) and grass pea (BioL 202 and Prateek) genotypes at untreated control, 30 µM arsenate, and arsenic + thiourea treatment. Data are means ± standard error of four replicates with different lowercase letters over error bars represent significant differences ($P < 0.05$) at ANOVA followed by Duncan's multiple range test

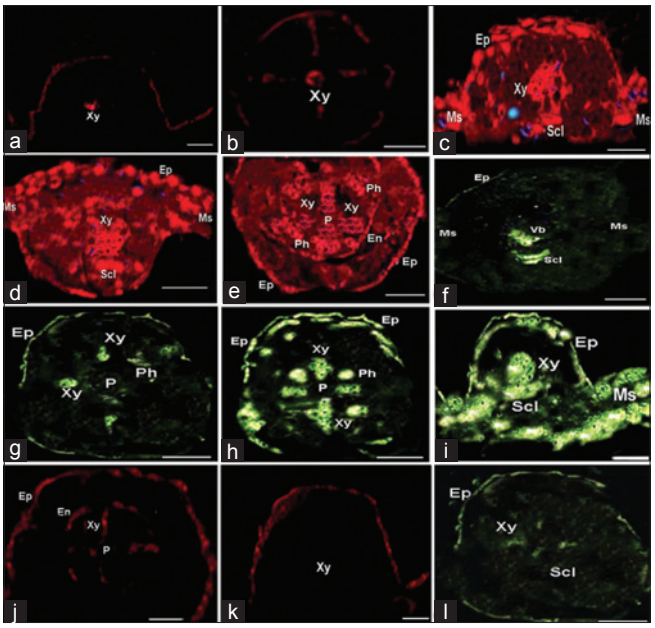

Figure 4: Representative photographs of reactive oxygen species imaging study by Confocal LASER Scanning Microscopy with dihydroethidium (superoxide radicals-specific) and 2'7'-dichloro fluorescein diacetate (H_2O_2-specific) dye showing mild red fluorescence in sections of (a) Control leaf, (b) control roots, bright red fluorescence in 30 µM arsenate (As)-treated (c,d) leaf sections (L 9 and K 75), (e) root sections of Pusa 4, mild green fluorescence in control (f) leaf and (g) root sections, bright green in As-treated (h) root, (i) leaf sections, reduced red fluorescence in (j) roots, (k) leaf and green fluorescence in (l) roots of Pusa 4 genotype after tetramethylpiperidinyloxy and ascorbate treatments (negative control). Blue region in Figure (c-e) indicates chlorophyll autofluorescence (chlorophyll A and B, λ excitation 429 and 450 nm; λ emission 650 and 670 nm). The results are representative of at least four independent experiments. Ep: Epidermis; En: endodermis, Ms: mesophyll cells; Scl: sclerenchyma; Xy: xylem; Ph-phloem; P-pith. Bars = 50 µm

(TF = 1) in all four genotypes but roots accumulated far greater amount than shoots (shoots/roots TF <1.0) in the presence of TU. The results suggested that TU application facilitated greater sequestration of As in crop roots and prevented upward translocation of As into photosynthetic parts. Comparing L 9 and Pusa 4, K 75, and PL 234 genotypes performed better, exhibiting higher growth traits but accumulating greater amount of As in their roots. The results pointed out genotypic differences for As accumulation pattern in present lentil crops.

As-induced growth inhibition may be related to severe reduction in photosynthetic apparatus as Chl A/B ratio, one of the significant biomarkers of plant photosynthetic ability, decreased due to reduction in Chl A level, and is significantly correlated with SDW in four genotypes. The reduction in carotenoids level suggested As-induced decline and/or damage in photosynthetic and antioxidant ability of the genotypes. The decrease in total Chl content is one of the early symptoms of metal (loid)-induced toxicity in crop plants (Azizur Rahman *et al.*, 2007; Talukdar, 2013b). Injuries in the photosynthetic apparatus result in many negative consequences such as reduction in plant growth, yield, and impediment in plant survival (Azizur Rahman *et al.*, 2007; Singh *et al.*, 2007; Ashraf and Harris, 2013). The improvement of plant growth by elevated dry mass accumulation and the sequestration of lion's share of As into roots by present genotypes after TU application was in fine tune with improved Chl A, Chl A/B ratio, and carotenoid content at As + TU treatment protocol. As-induced reduction in Chl A and Chl A/B ratio was observed in As-exposed edible legumes, cereals, and other crops but unlike the present circumstances in most of these cases carotenoid levels were not affected by As exposure (Guo *et al.*, 2005; Talukdar and Talukdar, 2013; 2014). The reasons for reduced photosynthetic pigment might be due to changes in chloroplast and carotenoid structure and/or inhibited biosynthesis of Chl/carotenoid or its precursors (Azizur Rahman *et al.*, 2007). Present results, thus, suggested strong ameliorative capability of TU in protection of photosynthetic pigment content which obviously had a positive effect on plant

Table 3: Changes in MG (μmol/g FW), H_2O_2 (μmol/g FW), MDA (nmol/g FW), and EL% in leaves and roots of L 9, Pusa 4, K 75, and PL 234 genotypes of lentils under untreated control, sodium arsenate (30 μM As), As+6.5 mM TU and As+13 mM TU for 10 days treatment duration

Traits	Treatments	Organ	L 9	Pusa 4	BioL 202	Prateek
MG	Control	Leaves	18.9±1.8[b]	20.4±2.0[b]	31.8±3.2[a]	33.6±3.7[a]
		Roots	13.7±1.4[b]	15.5±1.6[b]	19.7±1.9[a]	21.4±2.1[a]
	As	Leaves	23.6±2.3[b]	39.8±4.1[a]	40.7±4.0[a]	39.7±4.1[a]
		Roots	29.8±2.7[b]	43.2±4.3[a]	40.9±4.1[a]	45.8±4.6[a]
	As+6.5 mM TU	Leaves	18.3±1.7[b]	20.0±2.0[b]	30.3±3.3[a]	30.4±3.2[a]
		Roots	13.6±1.4[b]	14.5±1.5[a]	11.3±1.2[b]	17.7±1.8[a]
	As+13 mM TU	Leaves	8.7±0.9[b]	10.0±1.0[b]	14.8±1.8[a]	10.4±1.1[b]
		Roots	5.6±0.8[c]	6.8±0.7[b]	6.5±0.8[b]	10.5±1.1[a]
H_2O_2	Control	Leaves	4.3±0.4[a]	3.9±0.4[b]	3.6±0.4[b]	3.5±0.3[b]
		Roots	5.3±0.3[a]	5.4±0.5[a]	4.9±0.5[a]	4.9±0.5[a]
	As	Leaves	11.5±1.2[a]	9.8±0.9[a]	9.6±0.9[a]	10.5±1.1[a]
		Roots	10.8±1.1[a]	9.9±0.9[a]	9.8±0.9[a]	8.7±0.8[a]
	As+6.5 mM TU	Leaves	4.6±0.4[a]	4.1±0.4[a]	4.3±0.4[a]	3.3±0.3[b]
		Roots	4.9±0.5[a]	5.0±0.5[a]	5.1±0.5[a]	5.3±0.5[a]
	As+13 mM TU	Leaves	2.4±0.2[b]	2.8±0.3[a]	3.0±0.3[a]	2.7±0.3[a]
		Roots	2.1±0.2[b]	3.3±0.3[a]	3.2±0.3[a]	3.8±0.4[a]
MDA	Control	Leaves	4.6±0.4[a]	4.1±0.4[a]	3.7±0.3[a]	3.9±0.4[a]
		Roots	4.9±0.5[a]	4.9±0.5[a]	4.8±0.5[a]	5.1±0.5[a]
	As	Leaves	16.5±1.6[a]	10.7±1.1[b]	10.5±1.1[b]	10.8±1.1[b]
		Roots	10.7±1.1[a]	9.5±0.8[a]	9.4±0.9[a]	9.1±0.9[a]
	As+6.5 mM TU	Leaves	4.2±0.5[a]	3.9±0.4[a]	3.9±0.4[a]	3.9±0.4[a]
		Roots	5.1±0.4[a]	5.1±0.4[a]	5.3±0.5[a]	5.4±0.5[a]
	As+13 mM TU	Leaves	3.3±0.3[a]	3.2±0.3[a]	3.1±0.3[a]	3.6±0.4[a]
		Roots	3.2±0.3[a]	3.3±0.3[a]	3.5±0.4[a]	3.0±0.2[a]
EL %	Control	Leaves	3.8±0.4[a]	3.1±0.3[b]	3.7±0.4[a]	4.1±0.4[a]
		Roots	4.3±0.4[a]	4.3±0.4[a]	4.6±0.5[a]	4.6±0.5[a]
	As	Leaves	13.7±1.4[a]	11.0±1.1[a]	10.8±1.1[a]	11.1±1.1[a]
		Roots	16.9±1.7[a]	12.6±1.2[b]	11.9±1.1[b]	15.3±1.5[a]
	As+6.5 mM TU	Leaves	4.2±0.4[a]	4.3±0.4[a]	3.9±0.4[a]	4.4±0.4[a]
		Roots	4.5±0.4[a]	4.0±0.4[a]	4.4±0.4[a]	4.6±0.5[a]
	As+13 mM TU	Leaves	2.5±0.2[a]	2.9±0.3[a]	2.5±0.3[a]	2.6±0.3[a]
		Roots	2.8±0.3[a]	3.1±0.3[a]	3.0±0.3[a]	2.8±0.3[a]

Means±SE of four replicates treatment^{-1}. Means followed by different lowercase letters in rows are significantly different at $P<0.05$ by ANOVA followed by Duncan's multiple range test. MG: Methylglyoxal, MDA: Malondialdehyde, EL: Electrolyte leakage, TU: Thiourea, As: Arsenic, SE: Standard error

dry mass accumulation, as suggested by significantly positive correlation between SDW and Chl A/B ratio. This has immense agronomic significance as photosynthetic pigments are one of the most vital internal physiological factors, which are believed to be the targets of As-induced toxicity in crop plants (Stoeva *et al.*, 2005; Singh *et al.*, 2007; Talukdar, 2013b).

As-induced significant increase in SOD activity and its reduction on imposition of As + TU treatment protocol suggested that As-treatment alone stimulated enhanced generation of superoxide radicals in leaves and roots of four genotypes. In contrast, TU application declined superoxide generation. During dismutation, SOD generates H_2O_2, which is another ROS across cellular membranes (Neill *et al.*, 2002; Noctor *et al.*, 2012). In this case, roles of three prominent AsA-GSH cycle enzymes against As-stress were studied. As-induced significant decline in APX activity severely jeopardized H_2O_2 scavenging in all four genotypes with more severe effects on L 9 and Pusa 4 than that on K 75 and PL 234. This resulted in overaccumulation of H_2O_2 in both organs of As-treated crops. APX exclusively requires AsA as co-factor during ROS scavenging, the process which ultimately generates DHA (Asada, 2006). Low AsA pool in the As-treated plant organs particularly in roots might be due to reduced DHAR activity which regenerates AsA from DHA. Low DHAR activity also led to reduction in AsA redox which hovered around 0.5 in roots and 0.6 in leaves of the genotypes. The genotypic differences were evident from greater reduction of AsA and GSH level and their redox values in L 9 and Pusa 4 than those in K 75 and PL 234. Along with low AsA pool, decreased GR level failed to recycle enough GSSG to GSH. GR is a NADPH-dependent enzyme (Foyer and Noctor, 2011; Noctor *et al.*, 2012), and failure to regenerate GSH resulted in low GSH redox and high GSSG level in photosynthetic and underground parts of all four genotypes with greater impacts on L 9 and Pusa 4 than K 75 and PL 234 genotypes. Thus, the three redox couples, i.e., AsA/DHA, GSH/GSSG, and $NADP^+$/NADPH were badly affected with different magnitudes in four genotypes by As exposure alone. This accompanied by low GSTs and

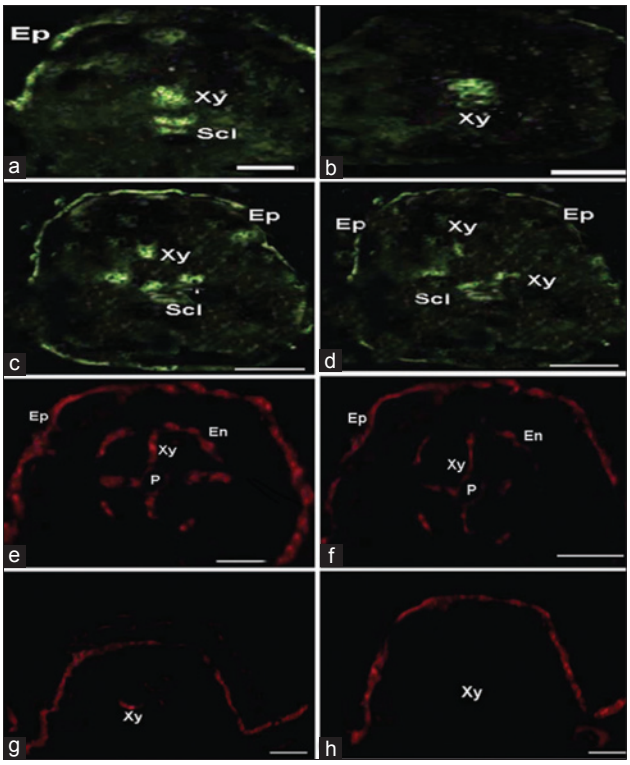

Figure 5: Representative photographs of reactive oxygen species imaging study by Confocal LASER Scanning Microscopy with dihydroethidium (DHE) (superoxide radicals-specific) and 2'7'-dichlorofluorescein diacetate (H_2O_2-specific) dye showing mild green fluorescence in arsenic (As) + 6.5 mM thiourea (TU) treated L 9 (a) leaf, (c) roots, As + 13 mM TU-treated L 9 (b) leaf, (d) roots, and mild DHE fluorescence in As + 6.5 mM TU-treated K 75 (e) roots, (g) leaf, and As + 13 mM TU-treated K 75 (f) roots and (h) leaf sections. The results are representative of at least four independent experiments. Ep: Epidermis; En: Endodermis, Ms: Mesophyll cells; Scl: Sclerenchyma; Xy: Xylem; P-pith. Bars = 50 µm

CAT level resulted in accumulation of H_2O_2 and lipid peroxides, which consequently led to membrane damage and EL as the marks of As-induced oxidative stress in the four lentil genotypes.

The antioxidant defense component was further crippled due to As-induced reduction in capability of Gly I and II systems. As and cadmium-induced inhibition of antioxidant defense and Gly system was noticed in different other crops (Hossain *et al.*, 2010; Hasanuzzaman *et al.*, 2012). Reduced capability of Gly system may have resulted in overaccumulation of MG and crippled regeneration of GSH which may be another reason, besides low GR, for low GSH redox pool in present genotypes exposed to As-treatment only. Present result confirmed interaction between GSH-dependent antioxidant defense and Gly system in lentil genotypes under As stress. GSH/Gly plays pivotal roles in primary antioxidant defense, as previously monitored in salt-stressed crop plants (Yadav *et al.*, 2005; Singla-Pareek *et al.*, 2003; 2008; Hossain and Fujita, 2010). In this study, exogenously applied TU significantly ameliorated As-induced oxidative stress by preventing excess build-up of H_2O_2 and MG, thereby protecting plants from As-induced oxidative damage. This was possible through modulation of primary antioxidant defense enzymes and Gly systems. In agreement with the present results, Gly system was found to be stimulated by exogenous GSH application in high temperature and drought stressed mung bean (Nahar *et al.*, 2015a; 2015b), by external NO in As-stressed wheat (Hasanuzzaman and Fujita, 2013) and by exogenous proline and betaine in drought-stressed mung bean and lentil seedlings (Hossain *et al.*, 2010; Molla *et al.*, 2014). In this case, significant reduction in SOD activity and elevation of antioxidant as well as Gly I and II activities under As + TU treatment suggested TU-mediated modulation of entire antioxidant defense and MG detoxification system in fine tune to prevent ROS and MG overaccumulation. Interestingly, AsA and GSH levels as well as activities of AsA-GSH cycle enzymes and GSTs along with Gly I and II increased significantly in roots of both crops at As + TU treatment regimes when As accumulated at the much higher amount in their roots than the shoots. Obviously, harmonious elevation of antioxidant activity and MG-detoxification system was required together to sequester and detoxify As in roots. High DHAR and GR level maintained AsA and GSH redox around 0.8-0.9, favorable to maintain cellular reducing environment. High GSH pool also favored activity of GSTs to be stimulated to scavenge lipid peroxide and to sequester As in roots of four genotypes. Furthermore, stimulated Gly I and II system in presence of TU helped lentil genotypes to contain MG level comparable to control. Thus, alteration of entire antioxidant defense and Gly system, and their integrated responses clearly indicated that despite huge As accumulation, roots of all four genotypes were able to detoxify ROS and MG quite effectively. This consequently protected photosynthetic parts from As-induced oxidative damage by preventing upward translocation of As and maintained plant growth under As exposure. The results indicated TU-mediated upregulation of defense cascades against As-induced oxidative stress.

Occurrence of superoxide radicals and H_2O_2 was imaged by CLSM using the fluorescence probe DHE and DCF-DA, respectively. Occurrence of faint red (superoxide) and green (H_2O_2) fluorescence in vascular bundle region indicated mild presence of free radicals in leaves and roots of untreated control. Contrastingly, bright fluorescence signals in vascular bundle regions, endodermis, sclerenchyma, mesophyll (leaf), and epidermis of As-treated leaves and roots of four genotypes

suggested overaccumulation and abundant localization of superoxide and H_2O_2 in plant parts. Image analysis suggested significant decline of ROS distribution in presence of 6.5 mM TU and further decline in presence of 13 mM TU. Significant taming of the fluorescence using TMP or AsA indicated specificity of DHE and DCF-DA for detection and imaging of superoxide radicals and H_2O_2, respectively, in crop tissues. Overproduction of superoxides and H_2O_2 due to toxic metals/metalloids was distinctly screened by CLSM study in pea, beans, alfalfa, and lupin roots (Ortega-Villasante *et al.*, 2005; Rodr´ıguez-Serrano *et al.*, 2006; Talukdar and Talukdar, 2013). This study pointed out effective roles of TU as ROS scavenger, and although MG could not be detected by present preparation, it is suggested that use of TU considerably reduced the magnitude of ROS production in leaves and roots of four genotypes.

CONCLUSION

The present investigation revealed As-induced oxidative stress and consequent inhibition of growth traits in lentil genotypes. The four genotypes, however, differed in their responses under As and As + TU treatment protocols. The L 9 and Pusa 4 exhibited higher impact of As-treatment than K 75 and PL 234. However, exogenous application of TU significantly improved growth performances by modulating As-accumulation pattern, Gly system, and antioxidant defense mechanisms coordinately in leaves and roots of As-treated genotypes. ROS imaging study confirmed As-induced ROS generation and its significant amelioration by exogenous application of TU in both plant organs.

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