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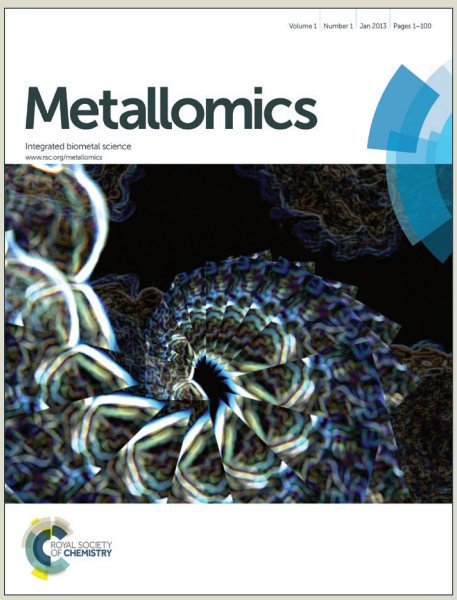

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Identification of redox-regulated components of arsenate (AsV) tolerance through thiourea supplementation in rice

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Abstract

Arsenic (As) is ubiquitously present environmental carcinogen that enters into human food chain through rice grains. In our previous research, thiourea (TU; a non-physiological thiol based ROS scavenger) application has been demonstrated to enhance salt and UV stress tolerance as well as the crop yield under field conditions. These effects were associated with TU ability to maintain plant redox homeostasis. Since, As stress also induces the redox imbalance, present research was initiated to evaluate TU efficiency for regulating As tolerance/accumulation in rice. The supplementation of TU (75 μM) to AsV (25 μM) improved the root growth and also reduced the As concentration by 56% from aerial parts that could be attributed to significant downregulation of Lsi2 transporter responsible the translocation of As from root-to-shoot. That these effects were not due to direct interaction between As and TU was confirmed from the complexation studies using HPLC-(ICP-MS)-(ESI-MS). The short-term kinetics study of GSH level and GSH/GSSG ratio confirmed the establishment of differential redox state in As and As+TU treated seedlings. The real-time RT-PCR based comparative expression profiling under As with/without TU treatment identified Sultr1;1 and Sultr1;2 as major redox-regulated sulphate transporters. Their specific induction in shoot coupled with enhanced root-to-shoot sulphate translocation (analyzed using ^{35}S-sulphate, as a radiotracer) was observed under TU supplementation. Further, the level of thiolic metabolites (PC2 in roots and GSH and PC3 in shoots) and activities of sulphur metabolism enzymes (ATP sulphurylase and cysteine synthase in roots and 5'-adenylylsulfate reductase in shoot) were also increased in As+TU as compared to As treatment. Thus, the study utilizes the interaction between As and TU to identify the critical redox regulated components of As tolerance in rice.

Keywords: Arsenic; phytochelatins; redox state; sulphate transporters; thiourea; tolerance.

INTRODUCTION

Arsenic (As) is ubiquitously present environmental toxin and recognized as group-1 carcinogen by International Agency for Research on Cancer (IARC). The health of nearly 150 million people worldwide from over 70 countries spanning six inhabited continents is threatened from As hazard. The major route of As contamination for humans is either through drinking water or crop and fodders[1], mainly rice[2]. Thus, different strategies are being developed to obtain low grain arsenic rice either through conventional breeding/varietal selection or by modern transgenics; however, these approaches will still take some time to come into use under field conditions. Under this milieu, the most potential strategy is supposed to be the management of agronomic practices to provide an immediate and sustainable solution to reduce As load in rice grains. Various approaches have been demonstrated to hold potential, e.g. growing rice with less irrigation[3], supply of silicate minerals[4] and phosphorus[5] and inoculation with arsenic-tolerant soil fungi[6] and mycorrhiza.[7]

Inorganic As is a prevalent form present in the environment, which exists as arsenate (AsO_4^{3-}, AsV) or arsenite (AsO_3^{3-}, AsIII), depending upon the pH and redox potential of environment[8]. Although the mode of toxicity of two As forms is different, As toxicity, in general, is associated with the induction of sulphur deficiency, oxidative stress and alteration in redox state.[9-11] Sulphur is an essential element for plant growth. There is a family of sulfate transporters (classified in group-1 to 4) which takes up sulphur in the form of inorganic sulfate.[12] Inside the plant, sulphate is first activated to adenosine-5'-phosphosulfate (APS) by ATP sulfurylase, and then reduced to sulfite by APS reductase (APR). Sulfite is reduced to sulfide, which is incorporated by cysteine synthase into O-acetylserine to form cysteine.[13] The key enzyme of sulphur assimilation pathway is APR which is regulated by transcription factor Long Hypocotyl 5 (HY5) in a demand driven and light-dependent manner.[14] The major proportion of

sulphur reduction takes place in shoot chloroplast which is supported by the light regulated nature of HY5.[15] Glutahione (GSH; γ-Glu-Cys-Gly) and phytochelatins (PCs; GSH oligomers) are the important sulphur-containing compounds responsible for As complexation, vacuolar sequestration and maintenance of redox state.[16-18] Importance of sulfur is also implicated by the fact that its supply affects As uptake, translocation and accumulation in rice plants.[19,20] The relevance of redox state in the regulation of As toxicity[9] and for the activation of downstream signaling event is known.[21] Thus, it was hypothesized that plant's As stress tolerance may be enhanced by avoiding the redox imbalance. In our earlier research, we have used thiourea (TU), as an external agent, to maintain the plant's redox balance under salt and UV stress.[22-23] TU is a non-physiological thiol and its broad range ROS scavenging activity under biological system is well documented.[24] The positive effect of TU was also demonstrated to enhance source-to-sink sucrose translocation[25], to identify the signaling and effector components of salt tolerance[26] and to improve crop yield and oil content of Brassica.[27] In the present work, effect of interaction between As and TU was utilized for the identification of redox regulatory mechanisms of As tolerance in rice. The efficacy of TU for reducing As load was also assessed.

MATERIALS AND METHODS

Plant material, growth condition and treatments

The study was performed on *Oryza sativa* var. IR64. Seeds were surface sterilized with 30% ethanol for 3 min and then washed thoroughly with distilled water to remove traces of ethanol. The seeds were then soaked in distilled water under shaking condition (~100 rpm) at 25ºC. The volume of water was adjusted so as to provide sufficient air to seeds while shaking. After 14-16 h of incubation, seeds were uniformly spread on a Petri plate and then allowed to

germinate under dark condition. A customized circular thermocol disc was made, which had a capacity to hold 18 seedlings. The 4 d old seedlings were fixed on these discs and then placed in 1 L beaker having 800 ml of ½ Kimura solution supplemented with different treatments such as AsV (prepared using the salt Na_2HAsO_4); As+TU and TU. One separate set was maintained as control. All the sets were transferred in plant growth chamber (Sanyo, Japan) having a daily cycle of a 14 h photoperiod with a light intensity of 150 µE m^{-2} s^{-1}, day/night temperature of 25/22°C and relative humidity of 65-75%. After 12 d of growth, differential phenotype was recorded in terms of dry weight/seedlings and average root and shoot length. Dry weights were measured after drying the samples to constant weight in an oven. The similar set-up was employed for the measurement of arsenic content, level of various thiols and activities of sulphur metabolism related enzymes. The root and shoot were harvested and stored at -80°C conditions till analysis. The harvesting time was fixed at 1 PM for each batch of experiment. For the measurement of short-term ^{35}S-sulphate uptake kinetics, redox couple (GSH and GSSG) and real-time RT-PCR based expression profiling, seedlings were grown for 15 d under control condition and then subjected to different treatments. For As+TU and TU, pre-treatment with TU was given for 24 h. In order to study the light-dependent regulation, the treatments were given at 9 AM and then 1, 4 and 8 h harvesting of root and shoot was performed and samples were stored at -80°C conditions until analysis. The concentrations of AsV and TU were 25 µM and 75 µM, respectively.

Arsenic measurement

For each treatment, seedlings were washed thoroughly in ice-cold milli-Q water to remove adsorbed As. The root and shoots were then separated and oven-dried at 80-85°C till constant dry weight. The dried tissue (~100 mg) was kept in 1 mL of concentrated HNO_3

overnight at room temperature and then digested at 120°C. The residue was then diluted in 10 mL of milli-Q water and subjected for As estimation using ICP-MS. The certified reference material (CRM) NIST 1568a rice flour from and blanks were included for quality assurance.

In vitro complexation studies of arsenic with glutathione and thiourea

To check the complexation of As with thiourea various combinations of As (4 to 40 mM, either As^{III} or As^{V}) and thiourea (33 to 330 mM), with and without GSH (3.3 to 33 mM) were tested. The substances were dissolved in degassed water or 0.1% formic acid and allowed to react 12-15 h under nitrogen. The complexes were analyzed through HPLC-(ICP-MS)-(ESI-MS).

The HP1100 HPLC system (Agilent Technologies Böblingen, Germany) with auto-sampler cooled to 4°C was used. The separation was done on a reverse-phase C18, Waters Atlantis column (150 mm x 4,6 mm x 5 µm, 100 Å) using a gradient of 0.1% (v/v) formic acid; A and 0.1% formic acid in 20% (v/v) methanol; B with a flow rate of 1 ml/min. Post-column, the flow was split in a ratio of 1:1 into the ICP-MS and ESI-MS. The 6130 quadrapole LC/MS system (Agilent Technologies Böblingen, Germany) was used as a molecule-specific detector for postcolumn detection of the As complexes by their molecular ion peaks. The MSD was used in the positive ionization mode from m/z 50 to m/z 1000 with API electrospray head. The settings chosen were: capillary voltage of 4,000 V, nebulizer pressure of 40 psi, drying gas flow of 12 L min^{-1} at 350°C, quadrupole temperature 100°C, and fragmenter voltage of 80 V. The ICP-MS 7500ce (Agilent Technologies Böblingen, Germany) was used for element-specific detection of As. The instrument was equipped with a microconcentric nebulizer (flow rate < 100 µL min-1), a Peltier cooled spray chamber, and oxygen as additional plasma gas. The instrument was used in the soft extraction mode. The instrument settings were checked daily for As sensitivity and optimized when necessary.

Fluorescence HPLC based estimation of various thiols

For the measurement of various thiols, liquid nitrogen ground plant samples (~400 mg) were extracted in buffer [diethylenetriamine pentaacetic acid (DTPA; 6.3 mM) and trifluroacetic acid (TFA; 0.1% v/v)]. The extraction was done on equal volume basis and supernatant was collected after centrifuging at 13, 000 g for 10 min at 4ºC. The supernatant (250 µl) was added with 615 µl of HEPES buffer [HEPES (200 mM), DTPA (6.3 mM; pH 8.2)]. To this mixture, 25 µL of Tris(2-carboxyethyl)phosphine hydrochloride (TCEP; 20 mM; as a disulfur reductant) and 10 µl of N-acetyl-L-cysteine (0.5 mM; as an internal standard) was added and the final mix was pre-incubated at 45ºC for 10 min in a water bath. This step is required to ensure that all thiols are in a reduced state so that maximum derivatization can occur. For monobromobimane (mBBr) based derivatization, 10 µL of mBBr (50 mM) was added and mix was incubated under dark in a water bath for 30 min at 45ºC. The reaction was terminated by the addition of 100 µL of acetic acid (10 mM). The derivatized samples were filtered with 0.22 micron nylon syringe filters and then stored at −20ºC for HPLC analyses. Separation and analysis of various thiols (GSH, cysteine and PCs) was carried on reverse phase HPLC (Waters, USA) with purospher RP-18e column (Merck) using a gradient of solvent A (99.9% Acetonitrile + 0.1%TFA) and B (89.9% Water + 10% Acetonitrile + 0.1% TFA) at a flow rate of 1 mL min^{-1} as described in Minocha *et al.*[28] Fluorescence intensity with an excitation wavelength of 380 nm and an emission wavelength of 470 nm was recorded using a fluorescence detector (Waters 474). The chromatograms were recorded and analyzed using Empower software.

Measurement of activities of sulphur metabolism related enzymes

The liquid nitrogen ground plant samples (~500 mg) were homogenized in extraction buffer (1 mL), squeezed through four layers of cheese cloth and then centrifuged at 12,000 *g* for

15 min at 4°C. The specific extraction buffer was used for each enzyme as described previously by Hartmann *et al.*[29] The detailed methodology for the measurement of enzyme activity is given as supplementary information S-1. The protein content in the sample was measured as per the protocol of Lowry *et al.*[30]

Measurement of redox state in terms of GSH/GSSG ratio

The level of reduced (GSH) and oxidized (GSSG) glutathione was determined fluorometrically using o–phthaldialdehyde (OPT) as a fluorophore by following the protocol of Hissin and Hilf.[31]

Short-term uptake kinetics using ^{35}S-Sulphate as a radiotracer

For ^{35}S-sulfate radiotracer uptake kinetics, the hydroponic solutions of the seedling given different treatments were supplemented independently with ^{35}S-sulfate (2 MBq/L). After 1, 4 and 8 h, the root and shoot parts were separately harvested and ^{35}S-sulfate levels were measured by scintillation counting. For scintillation counting, seedlings were removed from the radioactive solution and then rinsed with the ice-cold non labeled nutrient solution [3 times of 20 sec each]. Root and shoot samples were weighed separately and then digested in 10ml of HCl (1N) at room temperature. After 7 d, 100 µl of digested extract was mixed with 5 ml of scintillation cocktail [naphthalene (30 g), PPO (2 g), ethylene glycol (100 ml), methanol (50 ml) were mixed and volume made up to 500 ml with dioxane] and then counted on protocol 2 of TRI-CARB 2100 TR liquid scintillation analyzer (Packard, Canberra), as described previously[32]. The efficiency of the counter used was 95%.

Primer designing and real-time PCR based expression profiling of sulphate and arsenite transporter (low silicon 2; Lsi2)

All the primers used for real-time PCR were from exon-intron boundary and designed using web-based Quant-prime tool.[33] The details of the primers are given in supplementary

information S-2. Specificity of all primers was confirmed by sequence analysis of RT-PCR amplicons. The DNA-free total RNA was extracted using mirVANA kit (AM1560, Ambion). The 260/280 and 260/230 ratio of more than 2 and intactness of rRNA bands (28/18 s) in denaturing gel electrophoresis were considered as quality control of RNA to be used for further analysis. RNA (2 μg) was subjected to cDNA synthesis using Superscript III RT (18080-093; Invitrogen) following the manufacturers protocol. Real-time PCR was carried out using Rotor-Gene 6600 (Corbett Life Science; www.corbettlifescience.com). Reactions were set up by combining 10 μL of SyBr green PCR reaction mix (Sigma; S 4320) with 2.5 μL of 1:5 diluted cDNA templates, 1.5 μL each of forward and reverse primer (10 mM each), and 4.5 μL of PCR grade water (Sigma W 1754). For gene expression analyses, the reference gene (tubulin) and one target gene were analyzed per run, and reactions were carried out in triplicates for each sample. The following PCR protocols were followed: 95ºC for 15 min; 40 cycles of 94ºC for 20 s, 55ºC for 30 s, and 72ºC for 30 s followed by 72 ºC for 10 min and melting curve analysis. The data of the Ct value (cycle threshold) was calculated for target/reference gene for each treatment and respective control and then $\log_2$ expression fold difference was calculated using REST-384 version 2 software. For both up- and down-regulation, 1.5-fold change was set as cutoff to detect significant change in expression.

Statistical analysis

The experiments were carried out in a completely randomized design. All the experiments were repeated at least twice to check reproducibility. One–way analysis of variance (ANOVA) was done on all the data to confirm the variability of data and validity of results. Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments using statistical software SPSS 17.0.

RESULTS

Thiourea supplementation partially alleviated arsenic stress

The post-germination phenotyping was performed under different treatments to evaluate the effectiveness of TU supplementation. The analysis revealed differential phenotype of seedlings subjected to As with/without TU treatments (Fig. 1A). There was a significant reduction in root and shoot length by 46 and 21%, respectively under As stress as compared to control. The supplementation of TU increased the root length (Fig. 1B) and dry weight (Fig. 1C) by 42 and 13%, respectively as compared to that of As alone treated seedlings. No significant difference was observed length and dry weight of shoots between As and As+TU treated seedlings (Fig. 1B). The phenotype of the seedlings subjected to TU alone treatment was comparable to that of control (Fig. 1A-D).

Level of arsenic in different plant parts

In roots, the concentration of As was not significantly different in As (2710 μg g^{-1} DW) and As+TU (2825 μg g^{-1} DW) treatments (Fig. 2A). However, TU supplementation significantly reduced the As concentration in the aerial parts of rice seedlings. The As+TU treated seedlings showed 56% reduction in As concentration in shoots as compared to that of As alone treatment (Fig. 2B). By taking into account root and shoot dry weight data and As concentration, total As content in root and shoot (μg) was calculated. It was found that total root As content per plant increased from 4.07 μg in As alone to 5.65 μg in As+TU while total shoot As content per plant decreased significantly from 0.092 μg to 0.046 μg.

Lack of complexation between arsenic and thiourea

To check the possibility of As complexation with TU, an *in vitro* experiment was performed and analyzed through HPLC coupled in parallel to ICP-MS, the element specific

detector and ESI-MS, the molecule specific detector (Fig.3). The complexes of As with TU and/or GSH which could form are; As-TU_3, GS-As-TU_2, GS_2-As-TU, As-GS_3. The reaction mixtures containing As^V in all combinations and As^{III} without GSH showed only one peak in ICP-MS corresponding to inorganic As. However, the reaction mixture containing As^{III}, TU and GSH showed four As species in ICP-MS. ESI-MS showed strong signal at m/z of 75, 687, 865 and 994 corresponding to inorganic As, As^+-GS_2, GS_2-As-CysGly+H^+ and As-GS_3+H^+ for the ICP-MS peaks. None of the peak corresponding to As-TU complexes was detected in ESI-MS. Thiourea, reduced GSH and Oxidized GSH were also detected through ESI-MS showing signals at m/z 77, 308, and 613 respectively for $[M+H]^+$.

Thiourea treatment modulates the level of various thiols

The fluorescence HPLC based detection was performed for thiols such as cysteine and GSH (Fig. 4A) and phytochelatins (Fig. 4B). The level of most of the thiols was significantly increased in both root and shoot under As and As+TU treatment. In roots, the cysteine, GSH and PC4 contents were increased by about 10-, 2.4- and 22-fold in both As and As+TU treatments as compared to that of control. This was in contrast to PC2 which was specifically increased by 56-fold in As+TU as compared to that of As treatment. No significant induction in the level of PC3 was observed under any treatment (Fig. 4B). In shoot, the cysteine content was increased by 1.15-fold in both As and As+TU treatment as compared to that of control. In contrast, the GSH level increased by 1.8- and 2.8-fold in As and As+TU treatment, respectively, as compared to that of control. The level of PC3 was increased by 2.63-fold in As+TU as compared to that of any other treatment. The level of PC2 was found to be same in As and As+TU treatments, while that of PC4 was increased in As (1.7-fold) but decreased in As+TU (0.5-fold), as compared to that of control (Fig. 4B). In TU alone treatment, no significant change in the level of any thiol

was observed in roots (Fig. 4A), however in shoots, the cysteine, GSH and PC2 contents were significantly increased as compared to that of control (Fig. 4A, B). To measure the extent of As chelation by thiols (GSH+PCs), molar ratios of -SH to As (analyzed in fresh samples) were calculated.[34] The molar ratio of –SH to As was 0.109 and 0.122 for As and As+TU in roots. Hence, a maximum of about 3.6% and 4.1% As would be chelated by thiols in roots assuming a stoichiometry of three-SH to one As. In contrast, -SH to As molar ratios were very high in shoot for both As (27) and As+TU (76) treatment suggesting an excess of thiols and that all As may be chelated.

Activities of sulphur metabolism related enzymes

The activities of sulphur metabolism related enzymes such as ATP sulfurylase (APS), 5'-adenylylsulfate reductase (APR) and cysteine synthase (CS) were measured in root and shoot of seedlings subjected to different treatments. The APS activity was increased by 4- and 1.19-fold in As+TU treated root and shoot, respectively, as compared to that of control. In As and TU alone treatments, no significant difference in APS activity was observed in root as well as in shoot (Fig. 5A, B). The APR activity in shoot was decreased and increased by 45% and 77% in As and As+TU treatment, respectively than that of control (Fig. 5C). No APR activity could be detected in roots. The light mediated regulation of APR activity through might be responsible for its significantly low activity in roots, which could not be detected. The CS activity in roots was decreased by 70 and 20% under As and As+TU treatments, respectively as compared to that of control and TU treatments (Fig. 5D). In shoots, no significant difference in CS activity was observed under any treatment (Fig. 5E).

Thiourea mediates modulation in cellular redox state

In roots, under As stress, GSH content decreased in a time-dependent manner and the maximum decrease of 32% was observed at 8 h. In As+TU and TU alone treatments, GSH level remained lower than control till 4 h and a sharp increase was observed at 8 h (66% and 42% increase in As+TU and TU treatments, respectively, as compared to that of control; Fig. 6A). In contrast with GSH level, GSH/GSSG ratio was found to be higher in all treatments compared to control with the maximum being at 8 h when the ratio was 1.35-, 2.26- and 2.1-fold higher in As, As+TU and TU alone treatment, respectively (Fig. 6B).

In shoots, no significant difference in GSH level was seen till 4 h in any treatment. At 8 h, GSH level was increased by 2.25-, 2- and 1.58-fold in As, As+TU and TU alone treatments, respectively, as compared to that of control (Fig. 6C). The response of GSH/GSSG ratio was similar to that of GSH level in all treatments (Fig. 6D).

Differential translocation of sulphate from root-to-shoot: ^{35}S-Sulphate based radiotracer study

In roots, the ^{35}S-sulfate was progressively increased in a time dependent manner in all the treatments. The control roots showed the maximum uptake at 1 h while the minimum at 8 h. In TU-treated roots, initially the uptake was slow until 4 h and then, there was increase in ^{35}S-sulfate uptake at 8 h. In As and As+TU treatments, the level of ^{35}S-sulfate was almost same until 4 h. However at 8 h, the ^{35}S-sulfate level was increased by 1.12- and 1.91-fold, respectively in As in As+TU treatment as compared to control (Fig. 7A).

In shoots, initially at 1 h, the level of ^{35}S-sulfate was almost same in control, As and TU alone treatments but was slightly higher in As+TU treatment. With the increase in time, ^{35}S-sulfate uptake increased in all treatments. However, the uptake of ^{35}S-sulfate was lower in control and As treatments as compared to TU alone and As+TU treatments with the least being

in control treatments. At 8 h, the total ^{35}S-sulfate level in As+TU and TU alone treatment was increased by 3.42- and 2.96-fold, respectively as compared to control (Fig. 7B).

Expression profiling of different classes of sulphate (Sultr's) and AsIII (Lsi2) transporters in root and shoot

In roots, Sultr 1;1, 1;2, 2;1 and 3;3 were up-regulated in both As and As+TU treatments, however, the level of regulation was comparatively higher in As than in As+TU treatment. Besides, the higher level of expression was maintained till 8 h in As for Sultr1;1, 1;2 and 2;1 but not in As+TU. Additionally, few isoforms were regulated in a treatment-specific manner viz., the up-regulation of Sultr 1;3 in As+TU and Sultr 3;4 in As, at 4 h and Sultr 4;1 in As at 4 h and 8 h. In TU alone treatment, the level of most of the sulphate transporters was either significantly down-regulated or not significantly affected in roots on all time points except for Sultr 1;2 (at 1 h) and Sultr 3;3 (at 8 h) which were 2.19- and 2.48-fold up-regulated, respectively. The expression of Lsi2 was not changed under any treatment till 4 h of treatment. At 8 h, Lsi2 was downregulated by 3- and 2.5-fold, respectively in As+TU and TU treatments, as compared to that of control (Table-1A).

In shoots, under As stress, Sultr 1;1 and 1;2 were either down-regulated or remained at par to control except Sultr 1;1 at 1 h. This was in contrast to As+TU where the down-regulation of Sultr1;1 and 1;2 was limited to 1 h beyond which time-dependent increase was observed in their expression and the level at 8 h was 6.89- and 3.91-fold up-regulated for Sultr 1;1 and 1;2, respectively. The profile of remaining Sultr's responding at 1 h after treatment was also different between As with/without TU treatment. As treatment was associated with the induction of Sultr 2;1 and down-regulation of Sultr 3;2 and 3;3; while As+TU treatment caused up-regulation of Sultr's 1;3, 2;2, 3;1 and 4;1. In TU alone treated shoots, the profile of most of the Sultr's was

comparable to that of As+TU; however, the extent of change was significantly higher. At 4 h, Sultr 1;1, 1;2 and 2;1 were 1.21-,1.3-, and 1.41-fold higher in As+TU while 11.59-, 10.36-, and 7-fold higher in TU alone treatment. One isoform showing major difference in expression pattern between TU and As+TU was Sultr 3;4, which was up-regulated in TU (ranging from 0.75- to 1.51-fold at different time points) but not in As+TU (ranging from -0.58- to 0.24-fold at different time points) (Table-1B).

DISCUSSION

In an earlier research, TU supplementation has been demonstrated to impart salt tolerance through the maintenance of cellular energetics[35] and redox homeostasis.[22] Since, these are also the major determinants of As stress tolerance in plants[9,11], the present study was performed to evaluate the efficiency of TU for ameliorating As-induced damage and to implicate the significance of redox homeostasis in As stress tolerance. Initially, post-germination phenotyping of rice was performed on a range of As concentrations (5-50 μM) on the basis of average root length and IC_{50} value (25 μM) was calculated (data not shown). Then, a range of TU concentrations (10-200 μM) were tested along with 25 μM As (data not shown) and 75 μM TU was found to be optimum, which could partially revert the seedling phenotype (in terms of root length) (Fig. 1). The lack of complete phenotype reversal indicates that there are redox independent factors in As induced damage and hence, redox-homeostasis alone may not alleviate overall toxicity. Physiological thiol (GSH) has been evaluated in earlier studies for stress amelioration against As[36] and cadmium.[37] However, being a physiological thiol, it may also modulate a range of metabolic pathways in addition to redox state. Such a possibility is comparatively less for TU, which is a non-physiological thiol, and hence the observed effects can be correlated to redox state with a greater certainty. It has been confirmed in our earlier studies,

using HyPer-transformed Arabidopsis lines (Srivastava et al. Unpublished research) as well as through biochemical methods[22], that TU supplementation generates reduced redox state. TU-mediated shift in redox state towards reducing direction might be responsible for partial stress amelioration against As stress. As level was analyzed to test whether improved root growth in As+TU was associated with a decline in As. Surprisingly, As concentration in roots was not significantly affected. In fact, owing to the increase in root dry weight, the total root As content per plant in As+TU was even higher than As alone treatment. However, both As concentration and total shoot As content per plant were significantly reduced in shoots in As+TU as compared to As alone treatment (Fig. 2). This suggested that the loading of As into xylem for root-to-shoot transport is affected under TU treatment. To test this hypothesis, expression level of Lsi2 (a silicon or AsIII exporter) was analyzed in roots under different treatments. Owing to the localization of OsLsi2 to the proximal side of epidermal and endodermal cells, it is involved in the translocation of As from root to shoot.[11] Although, the present study deals with AsV, AsIII specific transporters were analyzed because, inside the plants, AsV has been shown to be rapidly converted into AsIII.[38] Under As+TU treatment, Lsi2 expression was downregulated in roots which might be responsible for decreasing As level from shoot. This is an interesting data which signify redox state as an important regulator of As uptake and translocation in rice. This is further supported by the findings of Liu et al.[39] and Duan et al.[40], where BSO (L-buthionine sulfoximine, a GSH biosynthesis inhibiter known for creating oxidized redox environment) treatment has been demonstrated to enhance root-to-shoot or shoot-to-grain As translocation in Arabidopsis and rice. Since, application of TU under field condition is already established; the present result of TU mediated reduction of root-to-shoot As translocation can have implication for reducing As load from rice grains.

Inspite of the decrease in As level, no significant difference in shoot growth was observed between As and As+TU treatment (Fig. 1). This might be either due to short duration of experiment or due to difference in As concentration not being enough to produce visible difference in shoot growth. In contrast, root growth was improved in As+TU than in As treatment despite the fact that As concentration was not significantly different between two treatments (Fig. 1A, B). There may be two possible reasons for this observation. Firstly, there might be improved tolerance against As toxicity through enhanced antioxidant potential. Such a mechanism has been suggested for TU supplemented *Brassica juncea* seedlings subjected to salt stress.[22] Secondly, the level of free As might be variable between the two treatments, which may be achieved through efficient vacuolar sequestration of As mediated through some unknown redox-dependent transporter or, by As complexation either by TU itself due to presence of thiol group (-SH) or by GSH and PCs. The possibility of As complexation with TU was evaluated *in vitro* using HPLC coupled with parallel ICP-MS and ESI-MS. The data obtained indicated that the formation of As-TU complexes was not feasible (Fig. 3) and was ruled out as one of the possible mechanisms for reducing free As levels in roots. The induction of *in built* tolerance mechanisms of As complexation via thiolic metabolites was then studied. Thiol metabolism is regarded as a major determinant of As tolerance[41] as well as As accumulation in plants.[39-40] The fluorescence HPLC based profiling of various thiols was performed in both root and shoot (Fig.4) and significant differences were observed for PC2 in roots and GSH, PC3 and PC4 in shoots between As and As+TU treatments. However, the molar ratio of total thiols (GSH+PC2+PC3+PC4)-to-As confirmed that the major portion of As would be present as non-chelated form in roots of both As and As+TU treatments. This indicated that positive effect of TU on root growth was not dependent upon GSH/PCs mediated improved As complexation. This

might be due to preference for long-term As storage, as uncomplexed As, similar to what has been demonstrated for seaweeds.[42] Thus, the possibility of a vacuolar transporter mediating the transport of uncomplexed As do exist as discovered in lower plant *Pteris vittata*.[43] In contrast, thiols were present in excess in shoot and all As might be present as complexed in both treatments. The higher levels of GSH and PCs may play a role as redox buffer. This was also evident from the significant accumulation of cysteine, GSH and PC2 in TU alone treatment. Although, GSH is an established redox buffer[17], the role of PCs in redox balancing is only emerging.[18] Further, the sulphur assimilation was also studied to explain the differential synthesis of GSH and PCs under different treatments. The significant increase was observed in the activities of APS and CS in root (Fig.5 A, D) and APR in shoot (Fig. 5C) in As+TU as compared to that of As treatment. This suggests that the regulations of these enzymes are also under the redox control. Previously, the redox-dependent regulation of APR has already been shown.[14]

Although, the chemical action of TU for scavenging broad range of biological ROS is well established[24], to have a measure of redox state kinetics of plants at initial stages of As stress, GSH level and GSH/GSSG ratio in rice seedlings were measured. The selection of GSH/GSSG ratio was done as it is considered as the major determinant of cellular redox state[17]. In As+TU and TU alone treated roots, GSH/GSSG ratio was significantly higher than that of As treatment on all time points. In contrast, differential redox state in shoot was seen only at 8 h after treatment wherein both GSH level and GSH/GSSG ratio were higher in all treatments as compared to control (Fig. 6 C, D). In order to correlate these changes of redox status with sulphur metabolism, measurement of sulphate uptake kinetics was performed under similar treatment condition using ^{35}S-Sulphate, as a radiotracer. The comparative analysis of ^{35}S-Sulphate level in As and As+TU treatment confirmed that root-to-shoot translocation of sulphate

rather than its uptake is the rate limiting step behind As mediated induction of sulphur deficiency.[44-45] Further, the differential translocation observed under As with/without TU also confirmed that the process is redox regulated. In order to identify the associated candidate genes, the quantitative real-time PCR based comparative expression profiling of sulphate transporters was performed. In roots, the overall down-regulation of Sultr's in TU pretreated seedlings suggested their regulation in a demand driven manner.[12] However, the expression of Sultr 1;2, which is the major high-affinity sulphate transporter in plants, was increased at 1 h and not significantly down-regulated at 8 h in TU alone treatment that would have maintained the basal sulphate uptake. The improved plant's sulphur status under TU supplementation was also evident as the comparatively higher and extended expression level of selected group-1 (Sultr 1;1 and 1;2), -2 (Sultr 2;1), -3 (Sultr 3;3 and 3;4) and -4 (4;1) transporters were observed only in As treated roots and not in As+TU treatment. The expression profiling was correlated with radiotracer data, where the sulphate content in roots at 8 h under As was much higher than any other treatment (Fig.7A). The enhanced root-to-shoot sulphate translocation observed under As+TU and TU treatments was attributable to significant up-regulation of Sultr 1;1 and 1;2 in shoot. These results suggested the tissue-specific function for Sultr 1;1/1;2. In roots, they played a vital role in sulphate uptake while in shoot they were responsible for sulphate unloading to facilitate the root-to-shoot translocation. Apart from redox, these Sultr's were also found to be light-regulated as their enhanced expression was observed only after 9 AM. Light-dependent regulation of Sultr 1;2 has already been demonstrated[46]. This is justified as maximum sulphate assimilation occurs only during day time. The early induction (1 h) of Sultr 2;2 (low-affinity transporter), Sultr 3;1[47] and Sultr 4;1 (for vacuolar sulphate remobilization) transporters in shoot of As+TU treatment might have contributed towards higher sulphate content observed even at 1

h time point in comparison to other treatments. This was probably to compensate the down-regulation of Sultr 1;1 and Sultr 1;2 at 1 h and suggest transporters others than those of group 1 are not light-regulated, however this needs to be assessed further. The significantly different signature of Sultr's observed in root and shoot under As, As+TU and TU treatment suggest that their expression is co-ordinately regulated by plant's sulphur demand, redox status and light. Recently, the regulatory role of plant sulphur status[48] and redox state[49] has been established for model plant *Arabidopsis thaliana*. To the best of our knowledge, this is the first study where spatial-, temporal- and redox-regulation of Sultr's have been studied in rice.

In conclusion, the study implicates the importance of redox homeostasis for ameliorating the As stress in rice through the use of TU, a non-physiological thiol based ROS scavenger. Under As stress, TU supplementation mediated the redox balance that led to the down-regulation of transporters for As translocation (Lsi2) leading to reduction in As level from aerial parts. This was simultaneous with up-regulation of sulphate transporters (Sultr 1;1 and 1;2), enhanced root-to-shoot sulphate translocation and increased activities of sulphur assimilation related enzymes which ultimately result in partial amelioration of effect observed under As stress. Thus, the findings not only signify the importance of redox-regulatory mechanisms for enhancing plant's tolerance against As stress tolerance but also widens the range of TU application for reducing As load from rice grains.

SUPPLEMENTARY DATA

Supplementary data are available online.

Supplementary information S-1: Detailed methodology for the measurement of activities of various enzymes.

Supplementary information S-2: Details of the primers used for the quantitative real-time PCR of different sulphate transports in rice.

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FIGURE LEGENDS

Fig. 1: Differential phenotype of *Oryza sativa* seedlings. The rice seedlings were grown for 4 d under control condition and then subjected to different treatments such as control, arsenic (AsV; 25 μM); arsenic (AsV; 25 μM)+thiourea (TU; 75 μM) and thiourea alone (TU; 75 μM)for 12 d. Differential growth phenotype (A), average root and shoot length (B), average dry weight of root (C) and shoot (D) were analyzed. The data represents the mean ± SE of three biological replicates. The experiment was repeated twice to check its reproducibility. Different letters on bar graph have been put on the basis of LSD value derived from SPSS software (DMRT, $P < 0.05$).

Fig.2: ICP-MS based estimation of arsenic level. The rice seedlings were grown for 4 d under control condition and then subjected to different treatments such as control, arsenic (AsV; 25 μM); arsenic (AsV; 25 μM)+thiourea (TU; 75 μM) and thiourea alone (TU; 75 μM)for 12 d. The root (A) and shoot (B) were harvested and used for the As estimation. The data represents the mean ± SE of five biological replicates. The experiment was repeated twice to check its reproducibility. Different letters on bar graph have been put on the basis of LSD value derived from SPSS software (DMRT, $P < 0.05$).

Fig.3: *In vitro* complexation study of arsenic with glutathione (GSH) and thiourea (TU). HPLC-ICP-MS/ESI-MS chromatograms of the reaction mixture containing As(III), thiourea and glutathione. ESI-MS (Blue line) data in scan mode and ICP-MS m/z 75 (As) (Black line) data were measured in parallel. ICP-MS traces showed four species of As which correspond to inorganic As and various complexes of GSH according to m/z signal in ESI-MS, as indicated in the Figure. None of the complexes contained thiourea. The experiment was repeated twice to check its reproducibility.

Fig.4: Fluorescence HPLC based estimation of various thiolic metabolites. The rice seedlings were grown for 4 d under control condition and then subjected to different treatments such as control, arsenic (AsV; 25 μM); arsenic (AsV; 25 μM)+thiourea (TU; 75 μM) and thiourea alone (TU; 75 μM) for 12 d and HPLC based estimation of reduced glutathione (GSH) and cysteine (A) and phytochelatins (B) was performed. The data represents the mean ± SE of three biological replicates. The experiment was repeated twice to check its reproducibility. Different letters on bar graph have been put on the basis of LSD value derived from SPSS software (DMRT, $P < 0.05$).

Fig.5: Measurement of activities of sulphur metabolism related enzymes. The rice seedlings were grown for 4 d under control condition and then subjected to different treatments such as control, arsenic (AsV; 25 μM); arsenic (AsV; 25 μM)+thiourea (TU; 75 μM) and thiourea alone (TU; 75 μM)for 12 d. APS (ATP sulphurylase; A: root and B: shoot), APR (5'-adenylylsulfate reductase; C: shoot; no activity detected in roots) and CS (Cysteine synthase; D: root and E: shoot) activities were assayed. The data represents the mean ± SE of three biological replicates. The experiment was repeated twice to check its reproducibility. Different letters on bar graph have been put on the basis of LSD value derived from SPSS software (DMRT, $P < 0.05$).

Fig.6: Measurement of redox state in terms of GSH level and GSH/GSSG ratio. The rice seedlings were grown hydroponically for 15 d under control condition and then subjected to different treatments such as control, arsenic (AsV; 25 μM); arsenic (AsV; 25 μM)+thiourea (TU; 75 μM) and thiourea alone (TU; 75 μM). After 1, 4 and 8 h of treatment, GSH level (A: root; C: shoot) and GSH/GSSG ratio (B: root; D: shoot) were measured. For As+TU and TU alone, 24 h pretreatment of TU was also given. The data represents the mean ± SE of three biological

replicates. The experiment was repeated twice to check its reproducibility. Asterisks (*) have been put on the basis of LSD value derived from SPSS software (DMRT, $P < 0.05$).

Fig.7: Short-term ^{35}S-Sulphate uptake kinetics. The rice seedlings were grown hydroponically for 15 d under control condition and then subjected to different treatments such as control, arsenic (AsV; 25 μM); arsenic (AsV; 25 μM)+thiourea (TU; 75 μM) and thiourea alone (TU; 75 μM). All treatment solutions were supplemented with ^{35}S-sulfate (2 MBq/L). After 1, 4 and 8 h of treatment, the root (A) and shoot (B) were harvested and ^{35}S-sulfate level was measured by scintillation counting. For As+TU and TU alone, 24 h pretreatment of TU was also given. The data represents the mean ± SE of three biological replicates. The experiment was repeated twice to check its reproducibility. Asterisks (*) have been put on the basis of LSD value derived from SPSS software (DMRT, $P < 0.05$).

Table Legend

Table-1: Expression fold difference (Log_2) of different sulphate transporters (Sultr's) and AsIII specific transporters (Lsi2) measured using real time RT-PCR. Rice seedlings were grown hydroponically for 15 d under control condition and then subjected to different treatments such as control, arsenic (AsV; 25 μM); arsenic (AsV; 25 μM)+thiourea (TU; 75 μM) and thiourea alone (TU; 75 μM). After 1, 4 and 8 h of treatment, RNA was extracted from root (A) and shoot (B) and used for real-time RT-PCR. For As+TU and TU alone, 24 h pretreatment of TU was also given. The data represents the mean ± SE of three biological replicates. For both up (marked as red) and down (marked as green) regulation, 1.5-fold change was set as cutoff and was considered as significant change. The details of gene-specific primers are mentioned in supplementary information S-2.

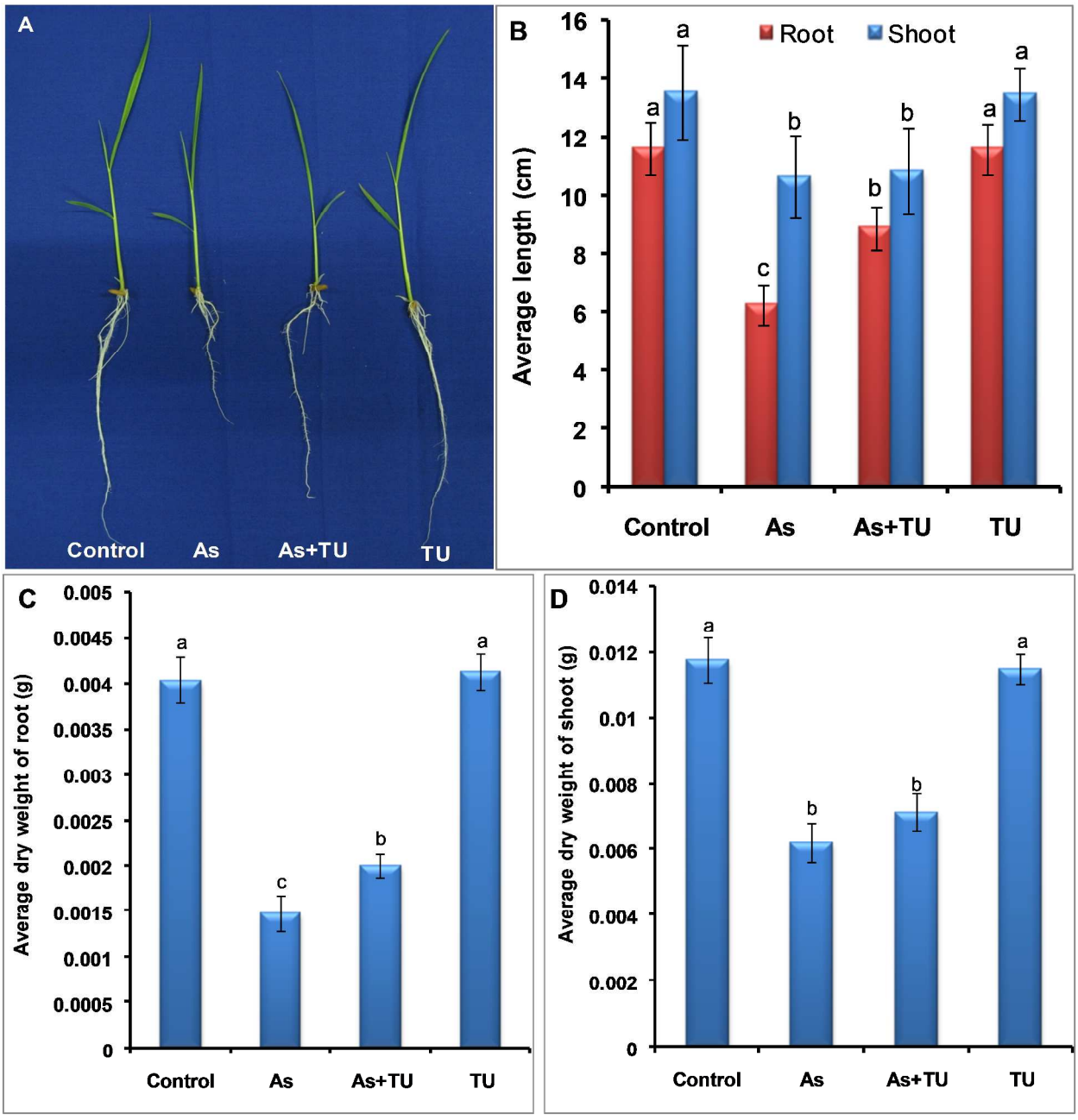

Fig. 1

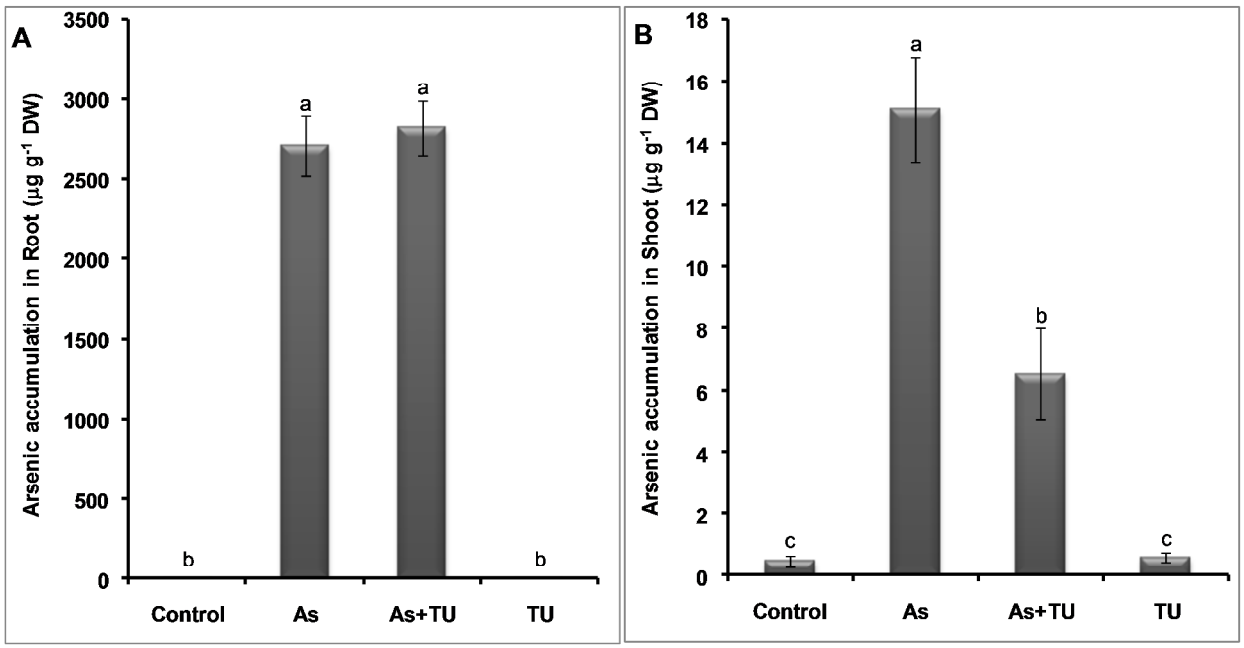

Fig.2

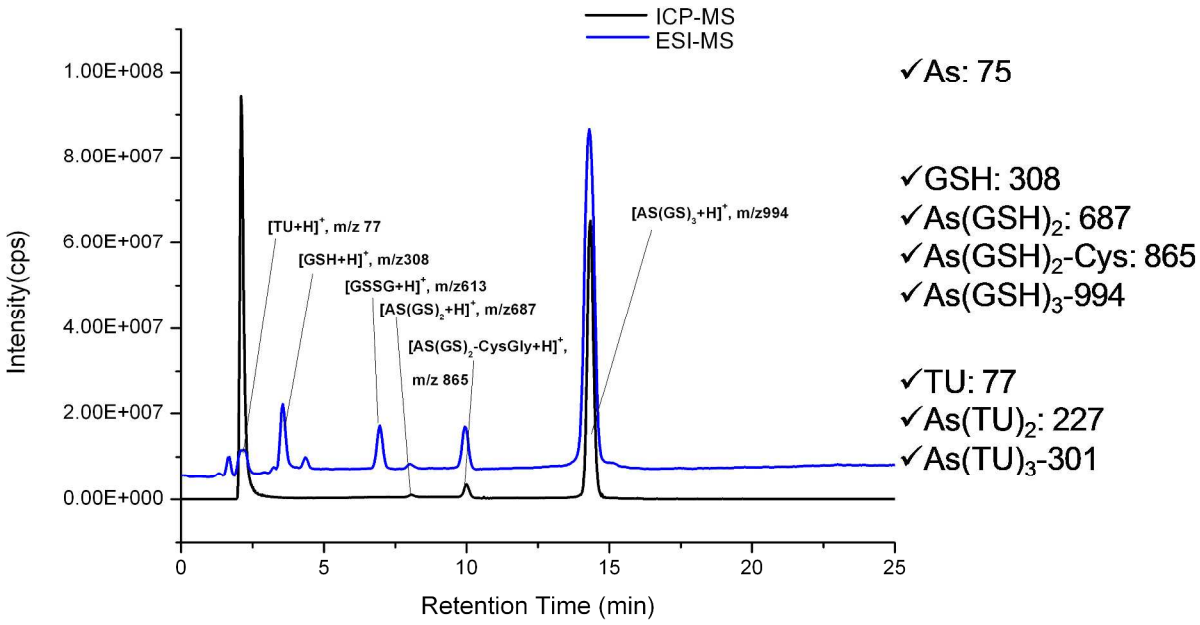

Fig.3

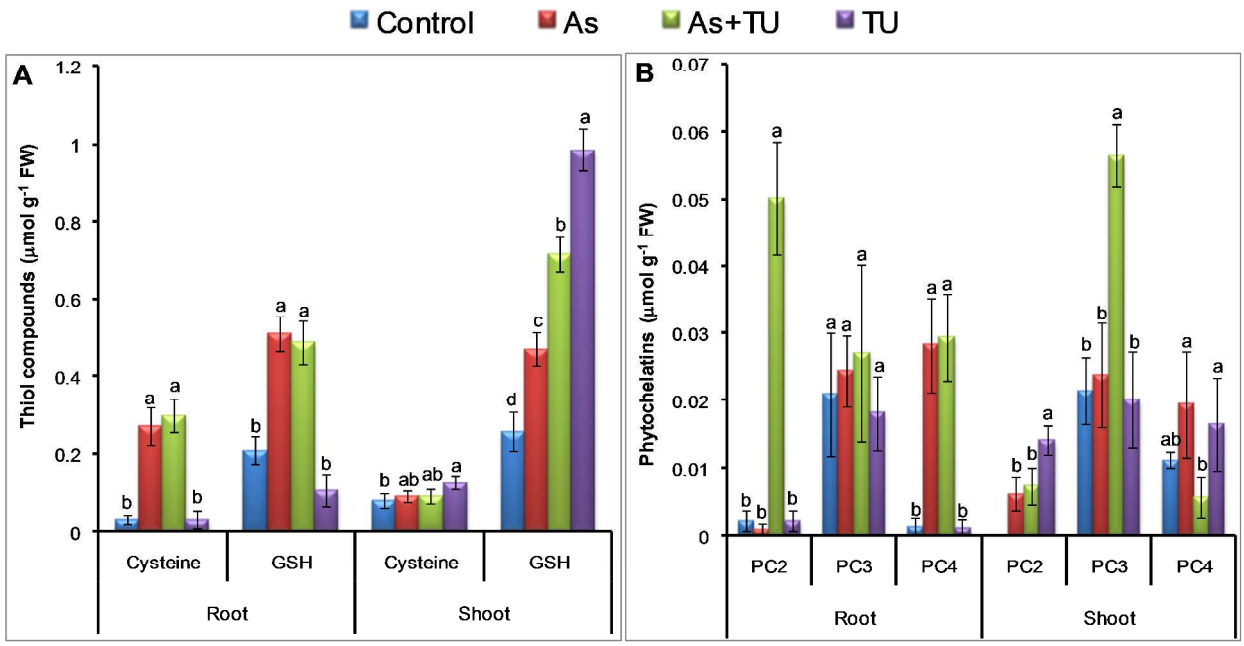

Fig.4

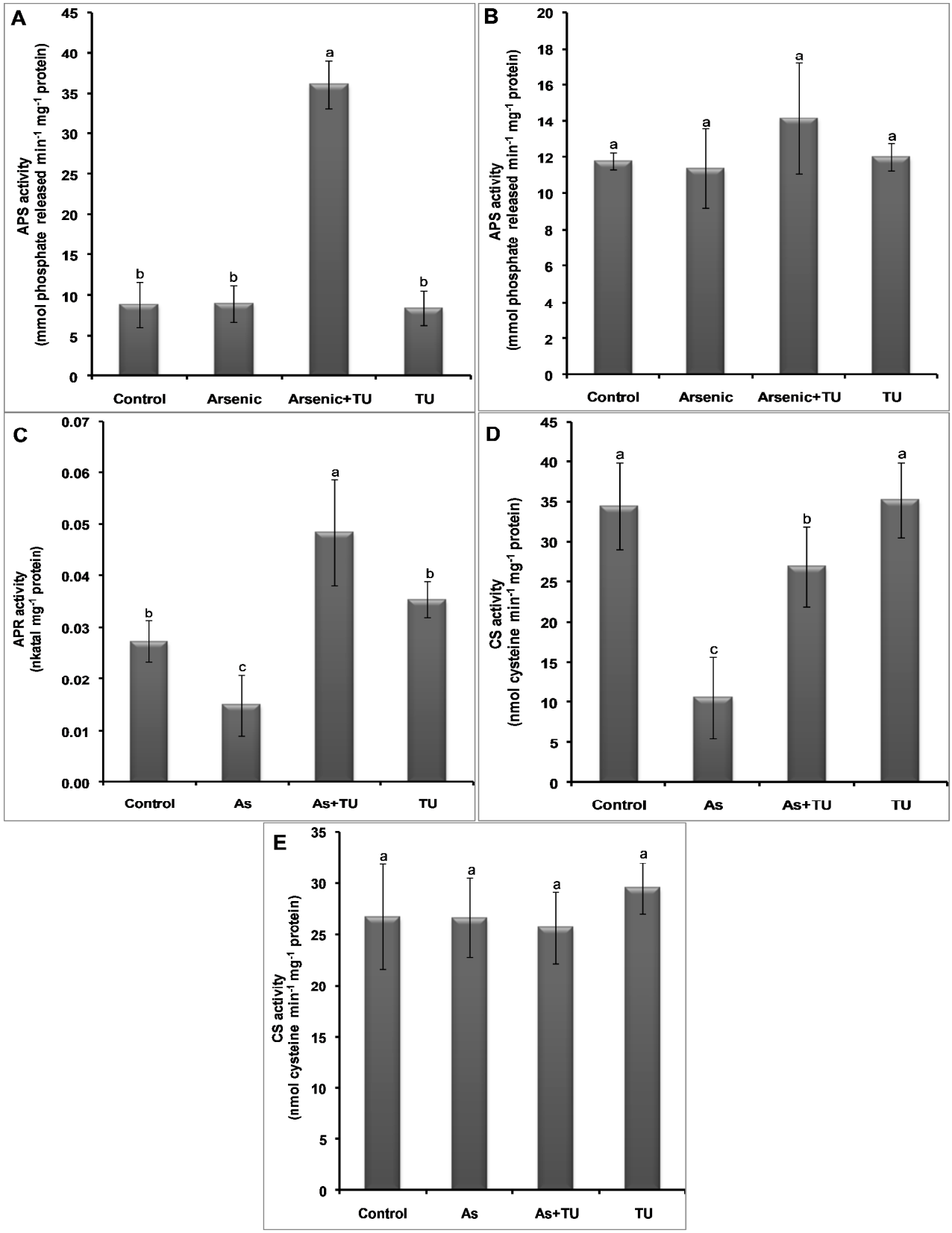

Fig.5

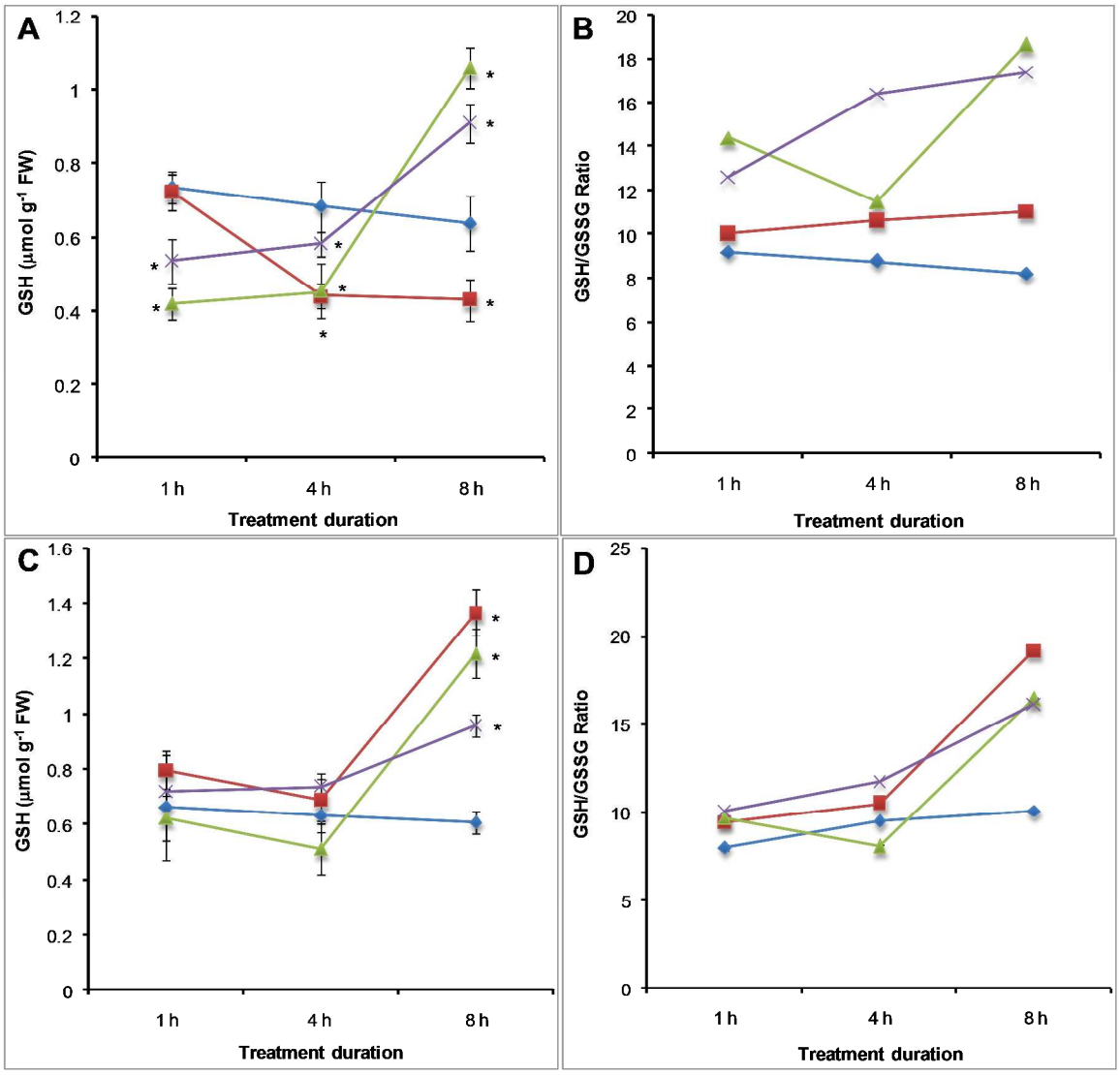

Fig.6

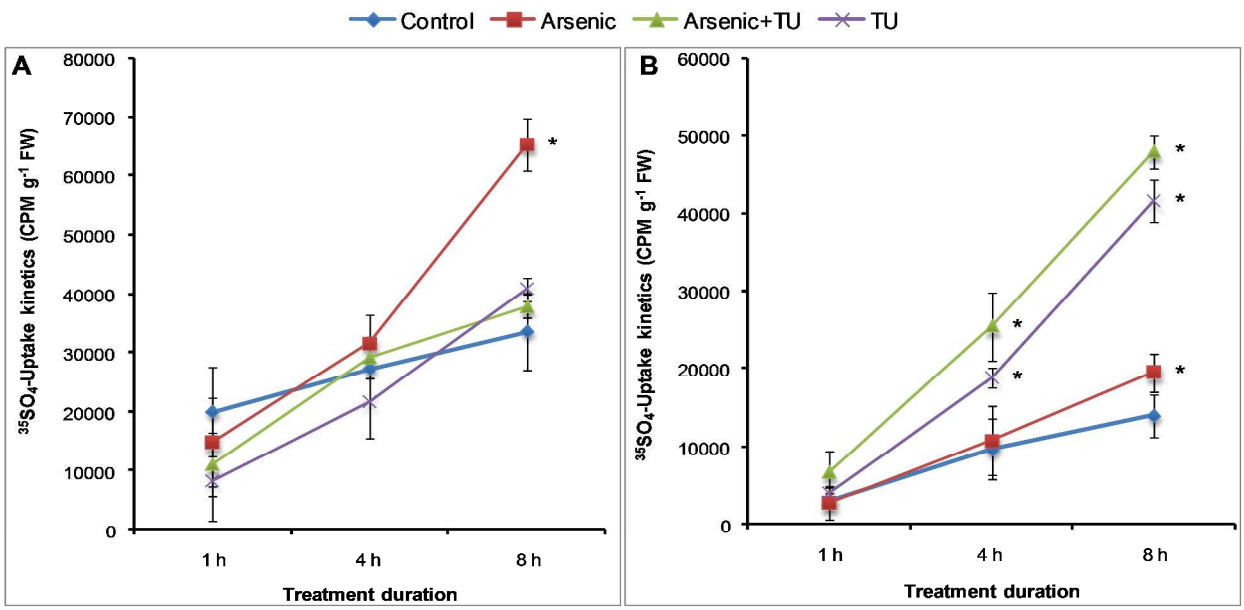

Fig.7

A

	Arsenic			Arsenic+TU			TU		
	1 h	4 h	8 h	1 h	4 h	8 h	1 h	4 h	8 h
Sulrt1;1	-1.43	4.467	3.859	1.135	3.733	1.306	0.95	-3.427	-3.058
Sultr1;2	-1.36	3.292	2.298	2.165	2.91	0.009	2.19	0.294	-0.867
Sultr1;3	0.34	1.332	-0.032	-0.325	1.985	0.419	-0.22	-0.931	0.158
Sultr2;1	0	5.641	3.636	0.26	3.553	0.612	-0.15	-1.616	-2.976
Sultr2;2	-1.16	0.972	-0.207	-1.82	0.03	-0.411	-1.145	-1.901	0.113
Sultr3;1	1.3	-0.548	-1.112	0.73	-0.975	-0.671	0.665	-2.351	-1.152
Sultr3;2	0.21	0.842	-0.277	-1.16	0.525	-0.381	-2.41	-1.516	1.283
Sultr3;3	1.05	2.157	-0.407	-0.555	1.74	0.754	-1.725	-1.756	2.488
Sultr3;4	-0.405	2.087	0.243	-0.9	0.99	-2.361	-1.375	-0.926	-2.127
Sultr3;6	0.05	0.75	0.143	-0.315	-0.47	-0.831	-0.945	-2.466	-1.597
Sultr4;1	0.94	2.587	2.273	0.545	1.435	-0.356	0.885	-2.001	-1.112
Lsi-2	-0.11	0.552	-1.481	-0.725	-0.94	-3.051	-0.385	-1.271	-2.472

B

	Arsenic			Arsenic+TU			TU		
	1 h	4 h	8 h	1 h	4 h	8 h	1 h	4 h	8 h
Sulrt1;1	3.88	-2.379	-0.451	-2.585	1.214	6.893	-6.21	11.597	8.17
Sultr1;2	0.275	-0.107	-0.114	-5.39	1.309	3.919	-8.37	10.36	5.037
Sultr1;3	-0.45	-1.142	0.346	1.835	-0.506	-1.541	1.775	-1.32	-1.643
Sultr2;1	3.445	1.563	0.248	0.95	1.413	1.015	0.485	7	1.379
Sultr2;2	-0.465	0.553	1.086	2.385	0.759	-0.271	1.715	-0.135	1.067
Sultr3;1	-0.43	-1.697	-0.324	1.995	-1.116	0.154	1.325	-1.295	-0.708
Sultr3;2	-2.01	-0.507	0.661	-0.335	-0.831	0.044	0.72	-1.46	-0.163
Sultr3;3	-2.46	-1.187	0.286	0.715	-0.516	-1.731	1.515	-1.995	-2.678
Sultr3;4	-0.505	0.568	-0.324	0.115	0.239	-0.576	0.755	1.695	1.512
Sultr3;6	0.25	-1.617	0.276	1.17	-0.886	0.869	0.18	-0.02	1.217
Sultr4;1	0.335	-1.787	-0.194	1.97	-0.951	0.354	1.06	0.87	-0.283

Table-1