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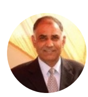

Arsenate Exposure Affects Amino Acids, Mineral Nutrient Status and Antioxidants in Rice (*Oryza sativa* L.) Genotypes

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Simulated pot experiments were conducted on four rice (*Oryza sativa* L.) genotypes (Triguna, IR-36, PNR-519, and IET-4786) to examine the effects of As^{V} on amino acids and mineral nutrient status in grain along with antioxidant response to arsenic exposure. Rice genotypes responded differentially to As^{V} exposure in terms of amino acids and antioxidant profiles. Total amino acid content in grains of all rice genotypes was positively correlated with arsenic accumulation. While, most of the essential amino acids increased in all cultivars except IR-36, glutamic acid and glycine increased in IET-4786 and PNR-519. The level of nonprotein thiols (NPTs) and the activities of superoxide dismutase (SOD; EC 1.15.1.1), glutathione reductase (GR; EC 1.6.4.2) and ascorbate peroxidase (APX; EC 1.11.1.11) increased in all rice cultivars except IET-4786. A significant genotypic variation was also observed in specific arsenic uptake (SAU; mg kg^{-1}dw), which was in the order of Triguna (134) > IR-36 (71) > PNR-519 (53) > IET-4786 (29). Further, application of As^{V} at lower doses (4 and 8 mg L^{-1} As) enhanced the accumulation of selenium (Se) and other nutrients (Fe, P, Zn, and S), however, higher dose (12 mg L^{-1} As) limits the nutrient uptake in rice. In conclusion, low As accumulating genotype, IET-4786, which also had significantly induced level of essential amino acids, seems suitable for cultivation in moderately As contaminated soil and would be safe for human consumption.

Introduction

Arsenic (As) is a carcinogenic metalloid, which can enter into the environment through natural as well as anthropogenic activities (*1*). Arsenic is a nonessential element for plant growth and has been reported to interfere with various metabolic processes that cause physiological and morphological disorders at higher doses leading to inhibited plant growth and death (*2*). Exposure to inorganic As, a non threshold class I carcinogen in populations not exposed to elevated As in drinking water is dominated by the consumption of rice (*3, 4*). Rice is a staple food for southeast Asian population. Since, irrigation of paddy fields with As contaminated groundwater has led to high As built up in soil (*5*) and rice is particularly efficient in As accumulation compared to other cereal crops (*6*) As^{III} being more mobile under anaerobic flooded conditions (*7*), elevated As levels in rice has emerged as a new disaster and matter of concern (*8*).

Selenium (Se) is a required micronutrient for humans with a recommended dietary allowance of approximately 0.9 μg kg^{-1} body weight (*9*), which is mainly fulfilled by the consumption of rice, which provides about 80% energy in some regions, proteins and micronutrients to the 50% population reliant on paddy rice for sustenance. Antagonistic effects or natural detoxification of As and Se have been reported in humans and other animals (*10*). Therefore, analysis of Se and other mineral nutrients vis-à-vis As accumulation may be helpful as this can help identify a suitable cultivar for human consumption.

Many studies have reported an enhanced generation of reactive oxygen species (ROS) upon As exposure, which can occur either directly by conversion of As^{V} to As^{III} or by disturbed redox balance in various ways (*11, 12*). To diminish the harmful effects of ROS, plants have evolved an effective scavenging system composed of antioxidant metabolites, such as GSH and enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidases (POD). Alteration in the activities of antioxidant enzymes and the level of metabolites in response to As have been studied in many terrestrial and aquatic plants (*11−14*) but very little information is available on effect of As exposure on antioxidants in rice (*15*).

Amino acids are the building blocks of proteins among which, histidine, proline, cysteine and glycine along with other amino acids are known to be induced significantly upon heavy metal exposure (*16*). Metal induced production of ROS may also modify amino acids leading to their loss. Proline has been reported to accumulate in tissues/organs of plants subjected to various abiotic stresses including heavy metal toxicity and appears to be a preferred organic osmoticum for many plants (*17*). Amino acids or protein content, along with other mineral nutrients in the food crops, will affect a great portion of the world population, especially in developing countries where rice grain is the main source of protein (*18*). Thus, quantification of various amino acids in response to different concentrations of As seems imperative in rice. Therefore, the underlying hypothesis was to test amino acids and mineral nutrients status with antioxidative (tolerant) characteristic of low grain arsenic accumulating cultivars. Thus, the present study was conducted to examine the effects of increasing levels of As^{V} on rice cultivars for As accumulation and its relationship with amino acids and mineral nutrient status in grain. Possible protective role of antioxidant enzymes were also investigated.

Materials and Methods

Soil Pot Experiment. The simulated pot experiment was conducted at Rice Research Station, Chinsurah, West Bengal, India, using four popular high yielding rice cultivars [Triguna, IR-36, PNR-519 and IET-4786 (Satabdi)]. Grains were surface sterilized with 0.1% $HgCl_2$ for 1 min, allowed to germinate

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in moist conditions and then transferred to 35 cm breadth × 25 cm depth pots containing 5 kg paddy field soil. Total six seedlings (2 plants/hill) were transplanted at three places in one pot. After transplantation, the pots were placed into polyhouse under natural light and humid conditions. Pots were watered daily with deionized water to maintain water logging condition. During tillering, preflowering and post-flowering stages the plants were irrigated with different As^V concentrations (0, 4, 8, and 12 mg L^{-1} As) using Na_2HAsO_4. The different concentrations were made in 3 L of deionized water and supplied twice in a day at the above-mentioned stages.

Amino Acid Analysis and Assay of Antioxidants. Amino acid analysis was done using HPLC by the pico tag method (detailed methodology provided in the Supporting Information). The activity of SOD, APX, GPX, catalase and GR were determined as standardized by the group earlier (*19*). Nonprotein thiols (NPTs) (*20*) were also measured for various treatments.

Quantification of Elements in Plant Parts and Soil. For estimation of Fe, Zn, and Se in soil and different plant parts (root, shoot, husk, and grain), samples were oven-dried at 70 °C and wet digested in HNO_3: $HClO_4$ (3:1 v/v). Protocol of Dwivedi et al. (*21*) was followed for digestion and estimation of total As. The level of Fe, Zn, Se, and As were quantified by inductively coupled plasma mass spectrometer (ICP-MS, Agilent 7500 ce).

Specific arsenic uptake (SAU) and specific selenium uptake (SSU) indicate the sum of total (T) As and Se uptake in different plant parts divided by root biomass (*22*).

$$SAU = (T_{\text{root-As}}(\text{mg}) + T_{\text{shoot-As}}(\text{mg}) + T_{\text{husk-As}}(\text{mg}) + T_{\text{grain-As}}(\text{mg}))/\text{root biomass(kg)}$$

$$SSU = (T_{\text{root-Se}}(\text{mg}) + T_{\text{shoot-Se}}(\text{mg}) + T_{\text{husk-Se}}(\text{mg}) + T_{\text{grain-Se}}(\text{mg}))/\text{root biomass(kg)}$$

Statistical Analysis. All the experiments were conducted following a randomized block design. Two-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments and genotypes. Correlation analysis was performed by following Gomez and Gomez (*23*). All the data of each cultivar with respect to change in metal content and between selected parameters, has been given within text at relevant places ($p < 0.001$***; $p < 0.01$**; $p < 0.1$*; ^{NS} nonsignificant).

Results

Physico-Chemical Characteristics of Soil. The pH of control soil was slightly alkaline, which decreased after repeated loading of 4, 8, and 12 mg L^{-1} of As (Supporting Information Table S1). The natural As concentration in control soil was low (5.43 mg kg^{-1} dw), which increased up to 31 mg kg^{-1} by irrigation of As^V (12 mg L^{-1} As dw). It was 20 and 14 mg kg^{-1} for 8 and 4 mg L^{-1} dw As^V treatments, respectively. The soil Se concentration was about 7 mg kg^{-1}. Though significant increase was found in available phosphorus, no changes were observed in the levels of other metals, that is, Fe, Zn, S, and Se.

Effect of AsV on Amino Acid Profile of Rice Grain. Rice genotypes showed differential response for amino acid (μ moles g^{-1} dw) composition under As^V stress (Figure 1A–D′). Interestingly a positive correlation was observed in total amino acids content in rice grain vis-à-vis As accumulation in rice genotype. The maximum concentration of total amino acids (Supporting Information Figure S5E) was observed at 12 mg L^{-1} As in IR-36 (1496, $R = 0.923$**), whereas it was the lowest for IET-4786 (897, $R = 0.795$*). In general, most of the essential amino acids significantly increased at 12 mg L^{-1} As, except IR-36, where only three amino acids viz., histidine, threonine, and isoleucine increased. Glutamic acid level decreased in Triguna (43%) and IR-36 (78%), whereas, a significant increase was found in IET-4786 (201%) and PNR-519 (85%) with increasing As^V concentrations. Alanine and arginine decreased in all the cultivars except IET-4786 where alanine increased by 85%. Moreover, a significant induction was observed in glycine in PNR-519 (35%) and IET-4786 (39%). Phenylanine (33%, $R = -0.841$*) and tyrosine (50%, $R = -0.780$*) decreased in IR-36 by 33%, ($R = -0.841$*) but increased in all other cultivars. A significant increase in cysteine was noticed in Triguna and PNR-519, whereas, IET-4786 showed an increase up to 8 mg L^{-1} As exposure. For IR-36, proline (93%, $R = 0.858$*) and aspartic acid (70%, $R = 0.870$*) increased significantly upon As accumulation, however cysteine level decreased at higher As^V treatments.

Effect of As^V on Antioxidant System in Rice. A significant induction in cysteine content of leaves was observed in Triguna (19%, $R = 0.948$**), IET-4786 (17.5%, $R = 0.967$**), IR-36 (17%, $R = 0.932$**) and PNR-519 (6.5%, $R = 0.608^{NS}$) upon As^V exposure (Supporting Information Figure S6F). Similarly, a concentration dependent increase in NPTs (Figure 2A) content was also observed in IR-36 (109%, $R = 0.981$**), Triguna (106%, $R = 0.991$**), and PNR-519 (57%, $R = 0.653^{NS}$), whereas, IET-4786 showed no significant effect.

Rice cultivars exhibited variable responses for antioxidant enzymes when exposed to As^V. A concentration dependent increase in SOD (Figure 2B) was noticed for Triguna ($R = 0.981$**), IR-36 ($R = 0.949$*), and PNR-519 ($R = 0.770^{NS}$), whereas a continuous decrease was observed in IET-4786 (48% at 12 mg L^{-1}, $R = -0.772^{NS}$). Similarly, GR (Figure 2C) also increased in Triguna (64%, $R = 0.984$**), IR-36 (35%, $R = 0.944$*), and PNR-519 (35%, $R = 0.844^{NS}$), whereas it decreased in IET-4786 (51%, $R = -0.915$*) with increase in As^V supply up to 12 mg L^{-1}. APX (Figure 2D) activity was decreased in IET-4786 (41%, $R = -0.932$*), whereas it increased in Triguna (69%, $R = -0.931$*) and IR-36 (40%, $R = 0.862$*) and PNR-519 (54%, $R = 0.707^{NS}$) with increasing As^V concentration. The activity of GPX (Figure 2E) decreased significantly upon all As exposures in Triguna (53%, $R = -0.897$*) and PNR-519 (60%, $R = -0.833^{NS}$), whereas no marked effect was observed in IR-36 and IET-4786. Further, all cultivars showed a similar trend for CAT (Figure 2F) activity, which declined during As exposure, maximum being in IET-4786 (48%).

Effect of As^V on Se Accumulation. Se accumulation differed significantly among the cultivars (Figure 3A–E). The maximum accumulation of Se (mg kg^{-1}dw) in roots (2.5; Figure 3A) and shoot (2; Figure 3B) was found in PNR-519 and Triguna, respectively. Supply up to 8 mg L^{-1} As increased the Se concentration in husk (Figure 3C) and grains (Figure 3D) in all cultivars. Triguna (0.64) accumulated highest amount of Se at 8 mg L^{-1}, whereas IET-4786 (0.12) showed lowest levels of Se accumulation in grains. The specific selenium uptake (SSU; Figure 4E) of four genotypes differed significantly and in general, the SSU of the rice cultivars enhanced with increasing concentration of As in the soil.

Arsenic Accumulation in Rice Parts. Total As (mg kg^{-1}dw) in roots (Figure 4A) was found to be maximum in Triguna (119) while minimum in IET-4786 (*14*) at 12 mg L^{-1} As exposure. Significant amount of As was transported from root to above ground parts and a considerable variation in As distribution pattern in above ground plant parts was observed among tested cultivars. The maximum level of As in above ground parts (Figure 4B–D) was exhibited by PNR-519 (ca. 16 at 8 mg L^{-1}) followed by Triguna and IET-4786 (ca. 15) and IR-36 (ca. 12) at 12 mg L^{-1}. Further, it was interesting to note that in IET-4786 most of the As was retained in shoot (Figure 4B) and husk (Figure 4C) and only 2–3% of the above ground As was transported to grains,

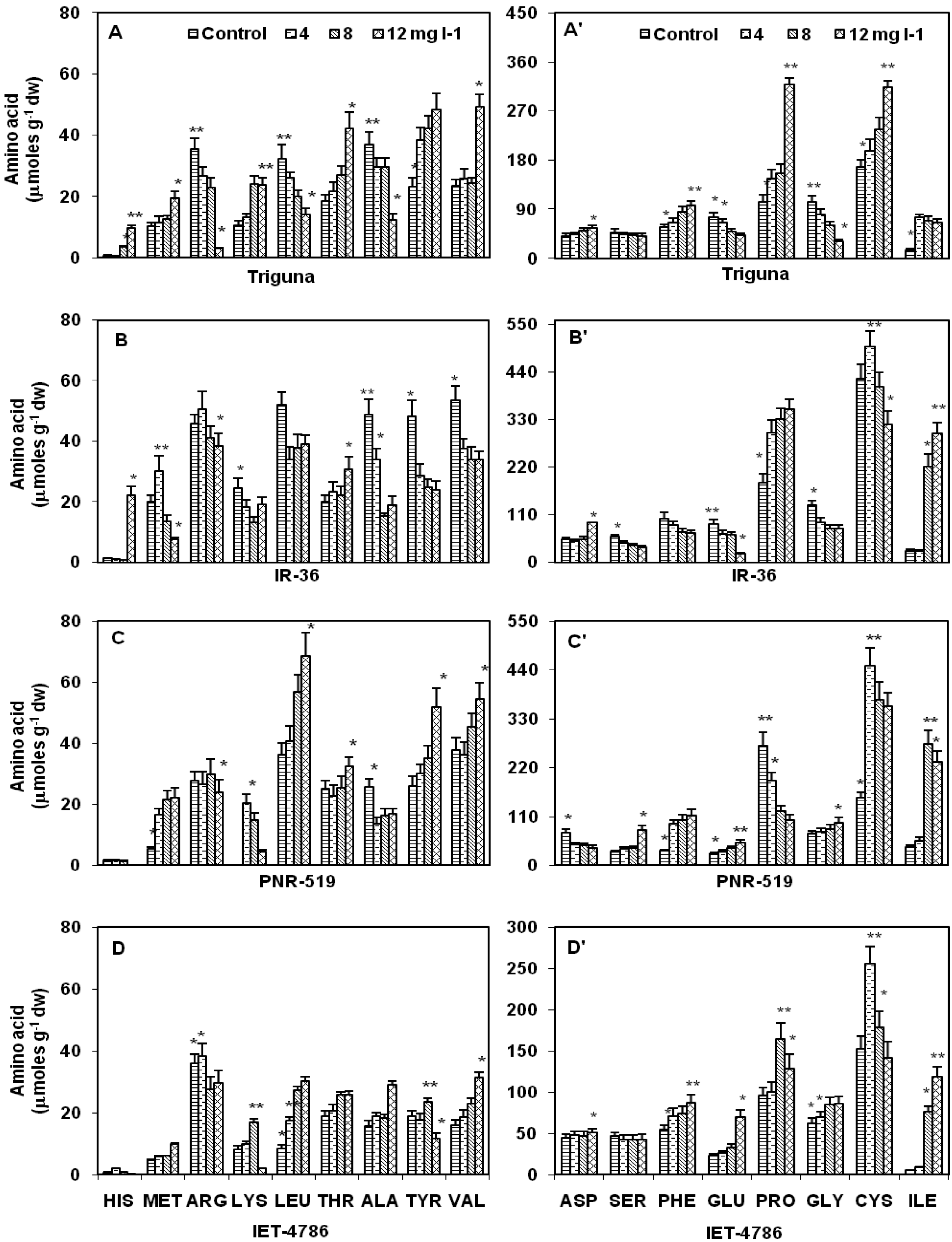

FIGURE 1. (A, A′–D, D′): Effect of As^V on amino acid profile in grains of rice cultivars. Triguna (A, A′); IR-36 (B, B′); PNR-519 (C, C′); IET-4786 (D, D′). All values are mean of triplicates ±S.D. ANOVA significant at $p \leq 0.01$. Star (*) indicates significantly different values (DMRT, $p \leq 0.01$).

which was significantly lower in comparison to other three cultivars (ca. 4−23% of above ground As). The maximum level of As in grain (Figure 4D) at 12 mg L^{-1} As exposure was found in IR-36 (ca. 1.5) followed by Triguna (ca. 1), PNR-519 (ca. 0.5), and IET-4786 (ca. 0.3). At this treatment a concentration dependent SAU was observed for various rice cultivars, which was highest for Triguna and lowest for IET-4786 (Figure 4E).

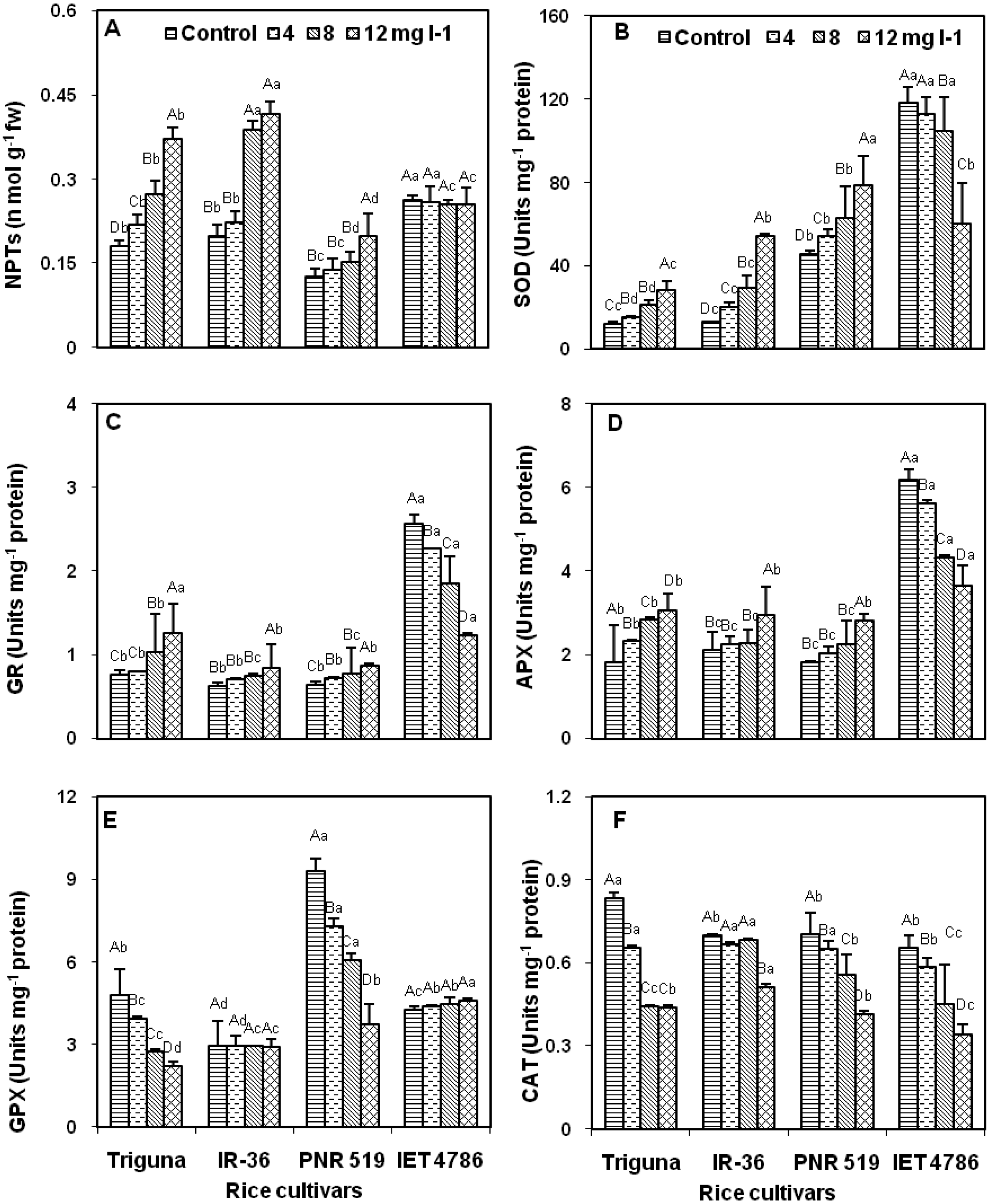

FIGURE 2. (A–F): Effect of As^{V} on NPTs content (A) and activities of SOD (B); GR (C); APX (D); GPX (E) and CAT (F). All values are mean of triplicates ±S.D. ANOVA significant at $p \leq 0.01$. Different capital letters indicate significantly different values among As treatments in a particular rice cultivar and small letters indicate significantly different values among rice cultivars at a particular treatment (DMRT, $p \leq 0.05$).

Discussion

The present study was conducted to examine the effect of As on amino acid profile, antioxidant defense responses and nutritional status of rice plants vis-à-vis As accumulation in pot conditions. As^{V} supply to soil strongly affected the amino acid profile of rice grains in a genotypic specific manner. Glutamic acid, glycine and cysteine which are components of GSH and PCs were induced significantly in IET-4786 and PNR-519 indicative of their low As accumulator nature. In non accumulator plants (*Helianthus annuus*) PCs have been found as a prevalent As detoxification mechanism (*24*). Proline is involved in plant heavy metal stress tolerance by different mechanisms such as osmo and redox regulation, metal chelation and scavenging of radicals (*25*). Its level increased considerably in the grains of Triguna, IR-36 and IET-4786 presumably due to the stress caused by As^{V}. Mishra and Dubey (*17*) observed a similar accumulation pattern of proline in As exposed rice seedlings. Further it is interesting that proline accumulation significantly decreased in PNR-519 at all the treatments which indicated a genotypic response in rice cultivars as observed for other parameters (*21*, *22*). Ni hyperaccumulator plant *Alyssum lesbiacum* accumulated higher level of histidine, proportionally to applied Ni exposure, demonstrating Ni−histidine complex in the xylem sap (*26*). Histidine and aspartic acid was induced significantly in Triguna and IR-36 in response to enhanced As accumula-

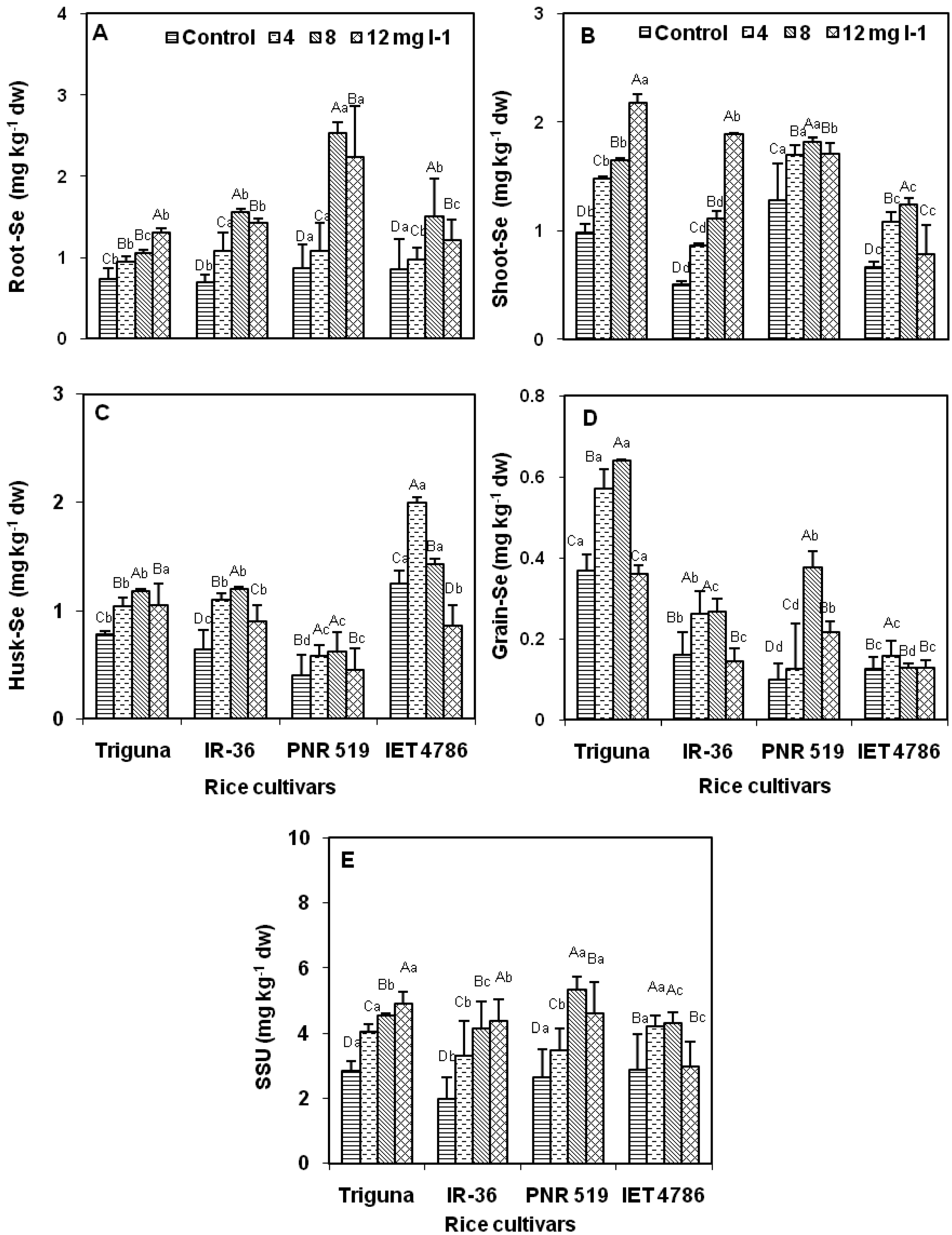

FIGURE 3. (A–E): Effect of As^{V} on Se accumulation in different parts of rice cultivars. Root Se (A); Shoot Se (B); Husk Se (C); Grain Se (D); SSU (E). All values are mean of triplicates ±S.D. ANOVA significant at $p \leq 0.01$. Different capital letters indicate significantly different values among As treatments in a particular rice cultivar and small letters indicate significantly different values among rice cultivars at a particular treatment (DMRT, $p \leq 0.05$).

tion. It may be likely that histidine and aspartic acid might be playing some role in As tolerance in rice as well.

Plants detoxify As by reducing arsenate to arsenite, which is subsequently detoxified via thiol reactive peptides such as γ-glutamylcysteine (γ-EC), glutathione (GSH) and phytochelatins (PCs) (*27*). NPTs, glutathione (GSH) and cysteine are important non enzymatic antioxidants. All cultivars showed a continuous increase in both cysteine and NPT contents (*12*). NPTs are considered as an index of GSH and PCs in plants (*28*), therefore, an increase in NPT contents of Triguna, IR-36 and PNR-519 upon As^{V} treatment may suggest an induction of GSH and PCs for protection of plant cells through direct chelation with As^{III} (*24*). IET-4786 did not exhibit a significant change in the level of thiols and this might be attributed to a higher basal level found in this cultivar (Figure 2A) to harmonize thiol biosynthesis and consumption (*29*).

The selected rice varieties have shown genotypic variations with respect to antioxidant enzymes. SOD plays an important role in dismutation of free superoxide radicals

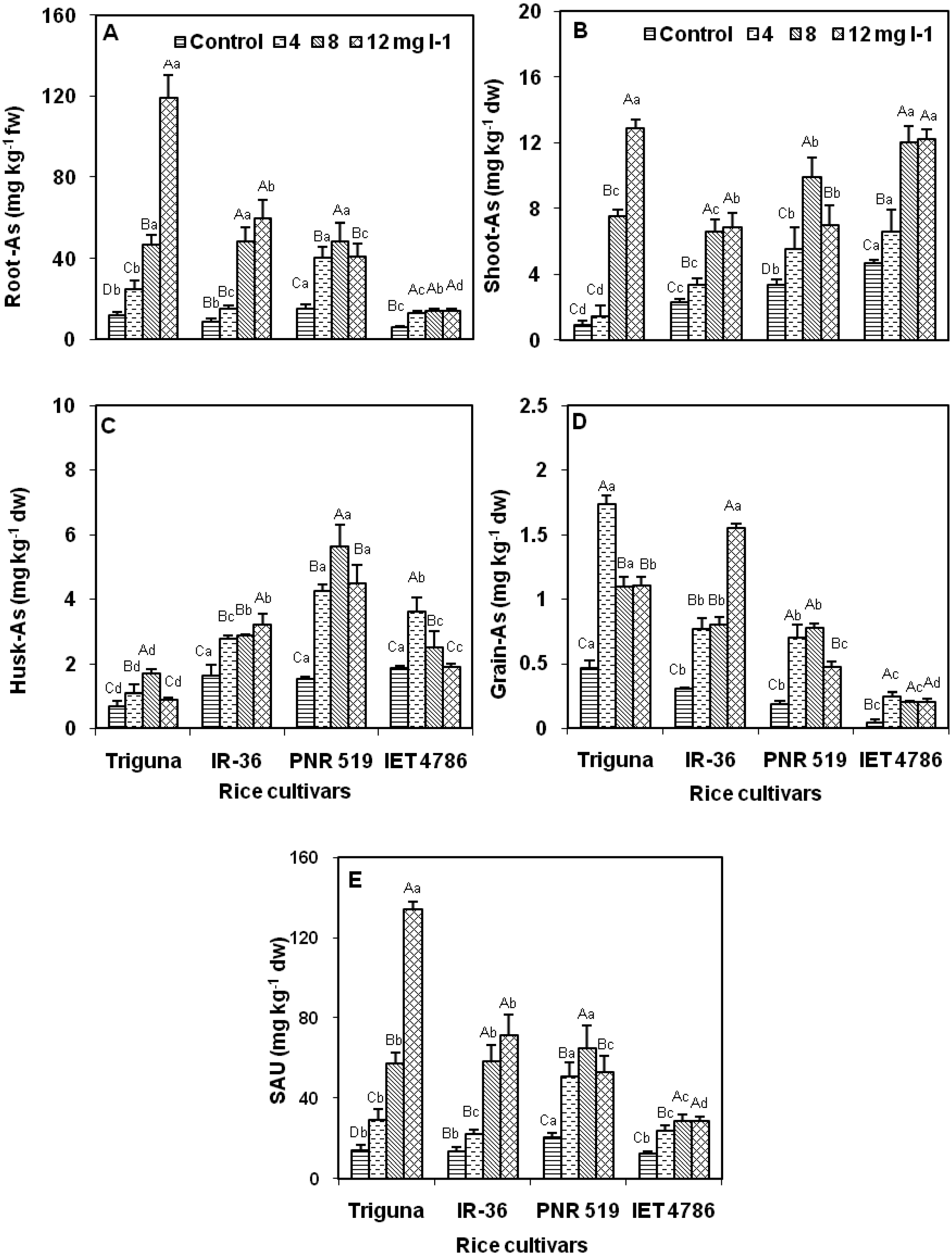

FIGURE 4. (A–E): Total As accumulation in different parts of rice cultivars. Root As (A); Shoot As (B); Husk As (C); Grain As (D); SAU (E). All values are mean of triplicates ±S.D. ANOVA significant at $p \leq 0.01$. Different capital letters indicate significantly different values among As treatments in a particular rice cultivar and small letters indicate significantly different values among rice cultivars at a particular treatment (DMRT, $p \leq 0.05$).

($O_2^{-\cdot}$). Significant increase in SOD activity was found in Triguna, IR-36 and PNR-519 while inhibition was observed in IET-4786 in response to As^V exposure, which may be due to impact on de novo synthesis of enzyme protein, as previously demonstrated in *Holcus*, *Ceratophyllum*, *Hydrilla*, *P. vittata*, and rice (*12*−*15*). Similar to SOD, GR activity also increased in Triguna, IR-36 and PNR-519 in response to As^V treatment. GR reduces GSSG back to GSH, which is crucial for maintaining redox balance of the cell. Oxidative stress is known to cause an increased GR activity (*28*), Significant induction in SOD and GR activity in Triguna, IR-36 and PNR-519 suggested that these cultivars responded positively to As stress by providing sufficient GSH to support antioxidant system viz., ascorbate-glutathione (ASC-GSH) cycle. APX activity also increased in Triguna, IR-36 and PNR-519 showing active participation of ASC-GSH cycle in these cultivars. However, Triguna and IET-4786 exhibited decreased APX activity. The other two peroxide degrading enzymes (GPX and CAT) showed no significant change in their activity. This might be due to less availability of H_2O_2 because of its efficient breakdown in ASC-GSH cycle (as in Triguna, IR-36, and PNR-519) or due to inactivation of enzyme directly by As or ROS.

Supply of As^V at low concentrations promoted yield by enhancing nutrient availability such as P, as observed by increase in number of tillers and seed weight (Supporting Information Figure S5A and D). Growth stimulation at low application of As has also been reported (*11*), whereas As hyperaccumulator *Pteris vittata* showed increased growth up to 100 mg kg^{-1} soil As application (*30*). In the present study, 12 mg L^{-1} As application retarded the growth and yield of rice cultivars as reflected by reduced plant height, number of tillers and seed weight, possibly due to retardation of chlorophyll contents as observed in the present study and also reported in oat (*31, 32*). Arsenic may inhibit chlorophyll biosynthesis by interfering the activity of δ-ALAD in greening maize leaf segments under As^V stress (*32*). Further, higher As concentrations, hampered uptake of essential nutrients such as Fe, Mn Cu, Zn, etc. may also be a reason for inhibition of photosynthetic pigments, (Supporting Information Figure S6A−D), reduced growth and number of tillers (Supporting Information Figure S5A−D) (*33*).

SSU of rice genotype differed significantly which was maximum (5 mg kg^{-1}dw) for Triguna and minimum (3 mg kg^{-1}dw) for IET-4786. Further, Triguna exhibited significant difference in Se accumulation in their root and shoot parts in comparison to IET-4786. These observations are in accordance with Zhang et al. (*34*) who reported significant difference in Se accumulation in shoot of japonica rice. Grain Se content varied remarkably (0.12−0.36 mg kg^{-1}dw, Figure 3D), which would fulfill the RDI of Se (55 μg per day). Recently, Williams et al. (*35*) suggested that Asian cultivars of rice are in general good Se accumulators.

The total amount of As accumulated in rice plants was highest in Triguna followed by IR-36, PNR-519, and IET-4786 indicating genotypic variation between rice cultivars (*22*). Dwivedi et al. (*21*) reported the highest residues of As in rice roots with intermediate values in shoot and husk, whereas grains showed the lowest levels. In the present study, IET-4786 with lowest SAU showed highest % As translocation to shoot while minimum As accumulation in grain, among the selected rice cultivars. However, the level of As in shoot of IET-4786 was still lower than those found in Triguna, which exhibited maximum SAU. Thus, Triguna and IET-4786 showed 5- and 4-fold differences in SAU and grain As accumulation, respectively (Figure 4E). Recent studies demonstrated that the As contamination in rice grain is elevated significantly (*1, 3, 21*) and its amount in grains depend on the baseline soil As levels (*5*) along with many genetic and environmental factors.

Based on our studies, it can be concluded that arsenic toxicity altered the amino acid content and antioxidant activity of the rice cultivars. IET-4786 accumulated less amount of As in grains leading to lower antioxidant profile and higher amino acids content. Thus, IET-4786 appears suitable for cultivation in moderately As contaminated soils. Further, the contrasting differences with respect to As acquisition and antioxidants between IET-4786 and other cultivars offer opportunity for breeding, aimed to select low grain As cultivar with desired productive and nutritional traits.

Acknowledgments

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Supporting Information Available

Methodology and results showing effect of As^V on Fe, P, Zn, S, photosynthetic pigments, total amino acids, cysteine, growth and yield of rice plants with physicochemical properties of soil (Table S1 and Figures S1−S6). This material is available free of charge via the Internet at http://pubs.acs.org.

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