RESEARCH ARTICLE

A Comparative Study to Alleviate Arsenite Accumulation in Hydroponically Grown *Oryza sativa* Seedlings Through Combinations of Se, P and Fe

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Abstract

In the present study, the nine cultivars namely NDR-3112, Swarn Sub-2, Pusa-sugandha, IPB-1, Pant-4, Pant-10, Jalnidhi, Ushar-3 and Mashina Research-2 were screened to select the As tolerant cultivar. Based on accumulation and growth parameters, the tolerant cultivar (cv. Pant 10) of rice was selected for further experiments. Rice cv. Pant 10 was treated with i) As, ii) As+Fe, iii) As+Se, iv) As+Se+P and v) As+P under hydroponic condition for 8d. The application (µg ml^{-1}) of Fe(100) to As(4) and Se(4)+P(6) to As(4), significantly (89% and 73%, respectively) reduced the uptake of As to the shoot without affecting the growth of seedlings. However, increasing the level of P (3 and 6 µg ml^{-1}) alone in As(III) (4 µg ml^{-1}) treatments significantly increased the As accumulation in the shoot, coincided with the decreased growth of rice seedlings. Treatment of As+Fe showed a non-significant reduction in growth relative to As alone, whereas, the seedlings exhibited the higher MDA and H_2O_2 level at 100 µg ml^{-1} concentration of Fe. In comparison to Se and P combinations, the Fe was found to be more efficient to significantly reduce the As accumulation. Among all the antioxidants, the activity of SOD and APX was significantly enhanced with the As+Fe(50) at both 4 and 8 days of treatments against As(4). Overall results demonstrate that the application of Fe was more efficient to reduce the uptake of As than Se and P in the rice seedlings.

Keywords: Arsenic, Iron, Phosphate, Selenium, Translocation.

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Introduction

Rice (*Oryza sativa* L.) according to an estimate is a staple food for 3.31 billion people out of the total 6.62 billion world population (Nguyen, 2006). The health risks associated with consumption of rice cultivated in arsenic (As) contaminated areas, is due to the translocation of inorganic As (iAs) to the grains. More importantly, the rice produced in European and Bangladesh/Indian regions contains 60 and 80% of inorganic As, in comparison to U.S. (42%) (Akinbile and Haque, 2012). Arsenic contamination in rice is a global concern and there are consistent efforts to minimize its translocation to the grains either by genetic modification or agronomic management to minimize its uptake. Among the two oxidation states of iAs, As(V) acts as a phosphate (P) analogue and can disrupt P metabolism in the plants, whereas, As(III) reacts with sulfhydryl group of several vital enzymes. Rice is speculated to accumulate higher amount of As(III) among all grain crops due to i) high phyto-availability of As(III) under reduced soil conditions (Wu *et al.,* 2011) and ii) being a aquatic plant, the presence of aquaporins on distal side of root, facilitates uptake of As(III). Arsenic is known to induce oxidative stress in the plants by generating reactive oxygen species (ROS) (Leterrier *et al.,* 2012), resulting in readjustment of transport and metabolic processes and growth inhibition. Plants have several mechanisms to defend themselves against the toxic effects of iAs. Compartmentalization by translocation of iAs to vacuoles is reported to be the basic mechanisms involved in iAs tolerance in plants (Abbas *et al.,* 2018).

Rice is typically an aquatic plant and Fe-hydroxides/oxides (plaque) are, ubiquitously formed on the roots of paddy rice (Xu and Yu, 2013). There are many reports on the interaction of Fe with iAs, where the As gets immobilized on the Fe-plaques formed on the rice root surface (Deng *et al.,* 2010). Similarly, the interaction of P reduces As in rice seedlings (Lou-Hing *et al.,* 2011). P deprivation in hydroponic condition enhances the formation of Fe plaque on the roots of rice seedlings while, P sufficient condition had little Fe plaque on its roots (Liu *et al.,* 2004). Hence, the inverse relationship between Fe-plaques on rice root and As uptake therein indicates a possible three-way interaction between Fe, P and As uptake. Selenium (Se) is an essential trace element and has been reported to have antagonistic effect on toxic elements in the plants (He *et al.,* 2004; Afton *et al.,* 2009; Feng *et al.,* 2009; Malik *et al.,* 2012). In cattles, treatment of iAs has been shown to prevent the Se induced toxicity by the formation of seleno-bis(S-gluthionyl) arsinium ion and eliminating it through urinary, biliary, and/or expiratory routes (Zeng *et al.,* 2005; Prince *et al.* 2007). Owing to their similar physical and chemical properties (i.e., similar valence shells, electronic structures, and atomic radii), iAs and Se compounds are speculated to be biologically antagonistic to each other. Rice mostly takes up $SeO^{2}_{3}^{-}$, which is the dominant form of available Se in the paddy soils and Se being a cofactor of the antioxidant enzyme glutathione peroxidase (GPX), hence it is required during oxidative stress (Zoidis *et al.,* 2018). According to a field study, negative corelation (R=-0.997) between grain As and Se accumulation was reported in 89

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rice cultivars when cultivated at three different sites in West Bengal (India) with variable soil-As level (Tripathi *et al.*, 2015). Selenium is also reported to take part in the metallothionin mediated conversion of GSSG to GSH , essential for countering ROS (Ruttkay-Nedecky *et al.,* 2013)

There are few recent studies which demonstrates to minimize the iAs uptake and translocation in rice by interaction with competiting ions i.e., As with Si (Tripathi *et al.,* 2013) Studies reported reduction of As uptake in rice cultivars owing to its adsorption on the Fe-plaques present on the root surface, whgich was higher in -P than in +P amended hydroponic medium (Liu *et al.*, 2004a; Lihong and Gulian, 2009). Liu *et al.* (2004a,b) showed that there was a significant correlation between the concentrations of Fe and As in iron plaque on the root surface of three rice genotypes which accounted for about 75-89% of total As. Recently, our study also reported that co-application of Se(IV) and P reduces As(III) and As(V) uptake and translocation in rice seedling shoots (Kumar *et al.,* 2013, 2016). While there are few reports on the interaction among Fe, Se and P relative to the uptake of iAs in plants, however, the combined effect of Se and P in reducing iAs uptake in rice, has not been investigated extensively.

Based on these reports and facts, it is hypothesized that As(III) and Se(IV) exhibit a antagonistic relationship in terms of their uptake in rice plant, as they share the same NIP transporters (Ma *et al.,* 2008), similarly, Se(IV) and PO_4 are also antagonistic in their uptake as they share same phosphate transporters (OsPT 1-14) (Li *et al.,* 2008). Hence, in presence of excess PO_4, the transport of Se(IV) across *Os*PT is compromised and the uptake of Se(IV) shifts to NIP transporters, thereby compromising the transport of As(III). Alternatively, Fe forms oxides as iron plaques on the O_2 emanating root surface of aquatic plants, thereby i) reducing the root surface area for uptake and ii) adsorbing the As(III) onto the root surface itself. Overall, all these three nutrients at interplay could eventually reduce the As(III) uptake. Hence, through this study an attempt has been made i) to assess the comparative effect on iAs translocation to the shoots of rice by combined addition of Fe, Se and P and ii) also to examine exclusively the role of Fe on iAs uptake and modulation of antioxidant defence mechanism in rice seedlings grown under hydroponic conditions.

Material and Methods

Germination and experimental design

Authentic seeds of rice (*Oryza sativa* L.) cultivars (9 nos.) namely; NDR-3112, Swarn Sub-2, Pusa-sugandha, IPB-1, Pant-4, Pant-10, Jalnidhi, Ushar-3 and Mashina Research-2 were procured from G.B. Pant University, Pantnagar, Uttarakhand and N.D. Agriculture University, Kumarganj, Faizabad, U.P. and were subjected for screening of the tolerant cultivar against iAs treatment. The seeds were surface sterilized with 10% H_2O_2 for 5 min followed by washing thrice with double distilled water. Seeds were germinated in seed germinator (G.G. Tech. Model: GG-123-EX) placed between moist blotting sheets on a sterilized plastic tray for 7d (dark) at 25°C and relative humidity (65%). After 7 days, the germinated seeds were placed under light 210 µmol cm^{-2} sec^{-1} (16/8h; day/night), produced by cold florescent light in a culture room maintained at 25±3°C, and were allowed to grow for next 15d. Uniform (10 cm) size seedlings (20 nos.) were placed in plastic cups (3.7cm inner dia., 5.0cm height) with netted bottom (3.5 cm diameter), these plastic cups containing the rice seedlings were then lowered in a dark coloured Perspex sheet containing 24 holes of the same diameter of the cups. The entire setup of cups fitted into the dark Perspex sheet was lowered into a plastic tray containing 2 liters of 100% Hewitt nutrient medium (Fig. 1) prepared in Milli-Q water (pH = 6.5-6.8) following Liu *et al.* (2004b). The plants were allowed to grow in the 100% Hewitt nutrient medium for 7d under light in culture room. Following the screening of the tolerant cultivar, treatment of As (AsIII), Fe (Fe^{3+}), Se (SeIV) and P (PO_4^{3-}) were provided to the screened/selected cultivar in desired concentrations using the salts of $NaAsO_2$, $FeCl_3$, Na_2O_3Se and KH_2PO_4 (Merck), respectively in the same assembly of cups in plastic tray. One separate tray was used for each treatment containing four replicates.

Screening of the nine cultivars of rice seedlings towards As toxicity was conducted against 1, 3 and 6 (µg ml^{-1}) of As(III) and it was observed that 6 µg ml^{-1} of As(III) caused severe toxicity on all the cultivars which were unsuitable for the study, hence, 2 and 4 (µg ml^{-1}) were selected for further studies. The cv. Pant-10 has shown higher accumulation of As in its shoot and therefore was selected for further study. Two independent experiments were performed in order to elucidate the effect of Fe, Se and P on the translocation of As(III) in rice. I) The seedlings were subjected to two concentrations of As(III) (2 and 4 µg ml^{-1} i.e., 26.66 and 53.33 µM, respectively) abbreviated as As(2) and As(4), respectively. Three concentrations of Fe i.e., 25 (448.02µM), 50 (896.05 µM) and 100 (1792.11 µM) µg ml^{-1} were provided with As (4) i.e., 4 µg ml^{-1} As with 25, 50 and 100 µg ml^{-1} separately, abbreviated as As(4)+Fe(25), As(4)+Fe(50) and As(4)+Fe(100), respectively. Similarly, 2 and 4 µg ml^{-1} of As with 2 (25.4µM) and 4 (50.7µM) µg ml^{-1} of Se separately, abbreviated as As(2)+Se(2), As(2)+Se(4), As(4)+Se(2), As(4)+Se(4), respectively. In order to elucidate the combined effect of Se and P on As uptake, 3 (63.33µM) µg ml^{-1} of P was applied with 2 and 4 µg ml^{-1} of both As and Se separately, abbreviated as As(2)+Se(2)+P(3), As(2)+Se(4)+P(3), As(4)+Se(2)+P(3), As(4)+Se(4)+P(3), respectively, 2 and 4 µg ml^{-1} of Se with 4 µg ml^{-1} As and 6 (126.7µM) µg ml^{-1} P, abbreviated as As(4)+Se(2)+P(6) and As(4)+Se(4)+P(6), respectively. Finally, 4 µg ml^{-1} of As with both 3 and 6 µg ml^{-1} P separately abbreviated as As(4)+P(3) and As(4)+P(6), respectively. II) Another experiment was conducted to study the effect of Fe on As, on the overall anti-oxidative defense mechanism and modulation of essential element uptake in the rice seedlings against the element where the reduction of As uptake was significant in a dose dependent manner. The seedlings were treated with As (4 µg ml^{-1}) supplemented with different concentrations of Fe i.e., 25, 50 and 100 µg ml^{-1}, abbreviated as As(4), As(4)+Fe(25), As(4)+Fe(50) and As(4)+Fe(100), respectively. Control plants were abbreviated as C. The seedlings were grown and treated in the same pattern as explained above, in separate plastic trays containing 2 liters of Hewitt nutrient solution (pH = 6.8) and harvested after 4 and 8 d for biochemical study. The experiment (Exp. I and Exp. II) consisted of 18 treatments including control which were carried out in 4 replicates in a total of 18 separate hydroponic trays. In order to minimize the error, the plastic cups were randomly placed in the trays. Throughout the experiment the nutrient solution was maintained to 2 liters supplementing with fresh Hewitt nutrient solution in order to maintain the evaporative losses. During the experiment the nutrient solutions were not aerated as rice was considered wetland plant and it has adapted to grow under anaerobic conditions.

Biochemical analysis, enzyme extraction and assays of the leaves

The lipid peroxidation was estimated by Heath and Packer (1968) and presented as thiobarbituric reactive substance (TBARS), H_2O_2

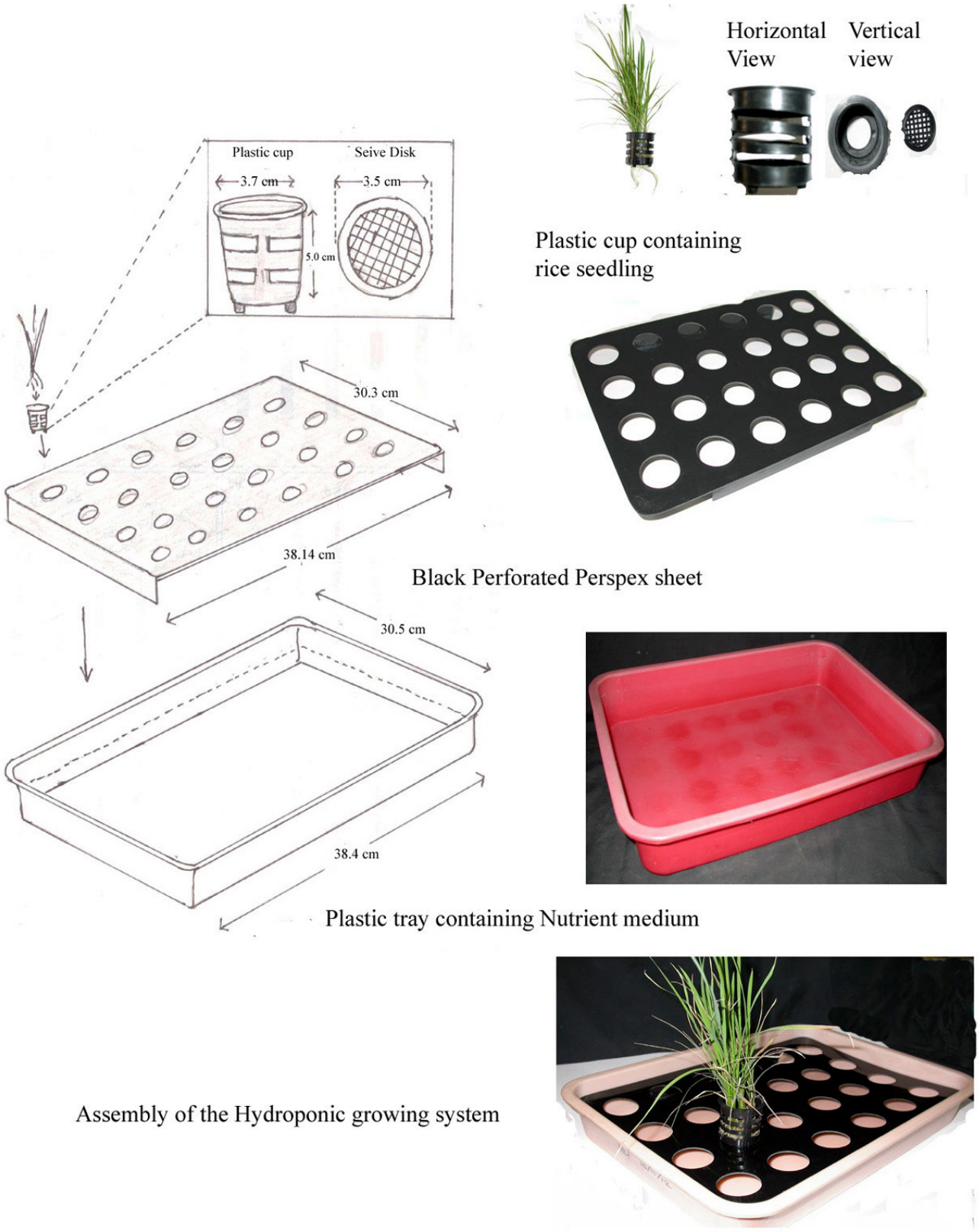

Fig. 1: Schematic diagram of home fabricated hydroponic growing assembly for rice seedlings.

content by Velikova *et al.* (2000), total soluble protein content by Lowry *et al.* (1951). For the analysis of antioxidant enzyme activities, fresh samples of leaves (300 mg) were ground using liquid nitrogen in a porcelain mortar and pestle which was extracted with 3 ml of ice-cold 100 mM potassium phosphate buffer pH 7.5, with 1% (w/v) polyvinylpyrrolidone. Homogenate was centrifuged at 12,000 RPM for 15 min at 4°C followed by storing the supernatant of the extract in an Eppendorf tubes (2ml) at -20°C. Small aliquots of the extracts were taken subsequently for anti-oxidative enzyme assays. The activity of superoxide dismutase (SOD) was measured spectrometrically at 560 nm following Beauchamp and Fridovich (1971) and presented as U mg^{-1} protein. 1 U of SOD activity is the amount of protein required to inhibit 50% initial reduction of nitro-blue tetrazolium (NBT) under light. The activity of ascorbate peroxidase (APX) was measured by Nakano and Asada (1981) by estimating the rate of ascorbate oxidation (ε = 2.8 mM^{-1} cm^{-1}) following Nakano and Asada (1981) and the enzyme activity was expressed as µmoles of ascorbate oxidized min^{-1} mg^{-1} protein. Guaiacol peroxidase (POD) activity was measured spectrometrically at 470 nm by Kato and Shimizu (1987) using its molar extinction coefficient (ε = 26.6 mM^{-1} cm^{-1}) and expressed as µmoles of guaiacol oxidized min^{-1} mg^{-1} protein, glutathione S-transferase (GST) by Droter *et al.* (1985) and expressed as mmol mg^{-1} protein, glutathione reductase (GR) by Smith *et al.* (1988) and represented as U mg^{-1} protein, where 1U is conversion of 1mM of oxidized glutathione (GSSG) to reduced glutathione (GSH) min^{-1}. Ascorbate oxidase (AO) activity by Oberbacher and Vines (1963) and represented as U mg^{-1} protein, where 1 U is oxidation of 1 µmol of the ascorbic acid min^{-1}.

Native polyacrylamide gel electrophoresis (PAGE) and activity stain

Leaf extracts (120 µg protein) were used for gel loading. PAGE was performed at 40°C, 25 mA, following Laemmli (1970). 10% polyacrylamide gel was used for resolving and 4% for stacking, omitting SDS form the PAG ingredients. Activity stain for APX was performed as Mittler and Zilinskas (1993), SOD by Beauchamp and Fridovich (1971) and POD by Fielding and Hall (1978). After electrophoresis, the gels were incubated in 25 mM potassium phosphate buffer (pH 8.0) for 15 min (POD basic), henceforth, the gels were submerged in freshly prepared solution containing 18 mM guaiacol and 25 mM H_2O_2 in 25 mM K-phosphate buffer (pH 5.0) (POD acidic) and till the GPX containing bands appear.

Elemental analysis

The plant tissues were acid digested following the method of Abedin *et al.* (2002). ~500 mg of finely chopped plant tissues (leaves and roots) were placed in 50ml beakers and HNO_3+H_2O_2 were added and left overnight, following which the beakers were placed on hot plate (60°C) in a fume-hood, and gradually the temperature was raised to 110°C till the tissues were completely digested and a white crust was left. The digested matter was dissolved by 2ml of Milli Q water (18mΩ) and filtered across whatman filter paper No. 42 and the filtrate were made up to 10ml by repeated dissolution and filtering with 2ml of water. Metal contents in digested samples were determined using AAS (GBC Avanta Σ) and As by Analyst 600 (Perkin Elmer) AAS (Mallick *et al.*, 2012). All the glass-wares and plastic-wares used were prewashed with 2% HNO_3 and dried.

Statistical analysis and Analytical quality control

The whole experiment was set up in randomized block design. The data were subjected to Tukey's test for the analysis of significant differences ($p<0.05$) between the treatments. Analytical data quality of the elements was ensured through repeated analysis (n=10) of Standard Reference Material. Standard certified reference material (CRM 028-050) used for the accuracy of the AAS was soil sample procured from Resource Technology Corporation, USA (Lot No. IH 028), and the values obtained (10 times) varied between -3.97 to 22.86% error (Table 1). The blanks were run all the time.

Results

Experiment I: Effect of the various treatments on the growth

Nine cultivars of rice were screened for the accumulation of iAs in the roots and shoots along with effect on the growth parameters (Fig. 2). It was observed that among all the cultivars (NDR-3112, Swarn Sub-2, Pusa-sugandha, IPB-1, Pant-4, Pant-10, Jalnidhi, Ushar-3 and Mashina Research-2), cv. Pant-10 exhibited the highest accumulation of iAs in the shoots (30.42 µg g^{-1} dw) and in the roots (784.68 µg g^{-1} dw) when treated with As (6 µg ml^{-1}) (Table 2). The growth parameters varied from one cultivar to another which decreased with increase in iAs concentrations, except in Pant-4 (Fig. 2). The two cultivars namely, IPB-1 and Pant-10 exhibited minimum decrease in growth parameters with increase in iAs concentrations. Based on the highest accumulation of iAs and minimum effect on growth parameters, cv. Pant-10 was selected for further studies.

The growth parameters decreased in cv. Pant-10 treated with As(4) which decreased significantly both in shoot (18.95%) and in root lengths (44.05%) over the C, however, the increase in biomass of the treated plants was not inhibited in same manner (Fig. 3). In As+Fe treated plants, non-significant difference in growth was

Table 1: Recovery results of the certified reference material (CRM 028-050) procured from Resource Technology Corporation (Lot No. IH 028) with As level 3.83 mg Kg^{-1}.

SN	*Wt. (g)*	*Final vol. (ml)*	*Dilution factor*	*µg Kg^{-1}*	*mg Kg^{-1}*	*% error*
1	0.302	10	6	16.131	3.204834	15.66225
2	0.3	10	6	15.31	3.36421	11.46816
3	0.295	10	6	14.34	3.84621	-1.21605
4	0.321	10	6	16.32	3.61021	4.994474
5	0.331	10	6	18.06	3.5275	7.171053
6	0.326	10	6	14.39	2.97536	21.70105
7	0.384	10	6	16.67	2.93108	22.86632
8	0.291	10	6	17.32	3.52731	7.176053
9	0.289	10	6	15.36	3.45164	9.167368
10	0.334	10	6	18.36	3.95109	-3.97605

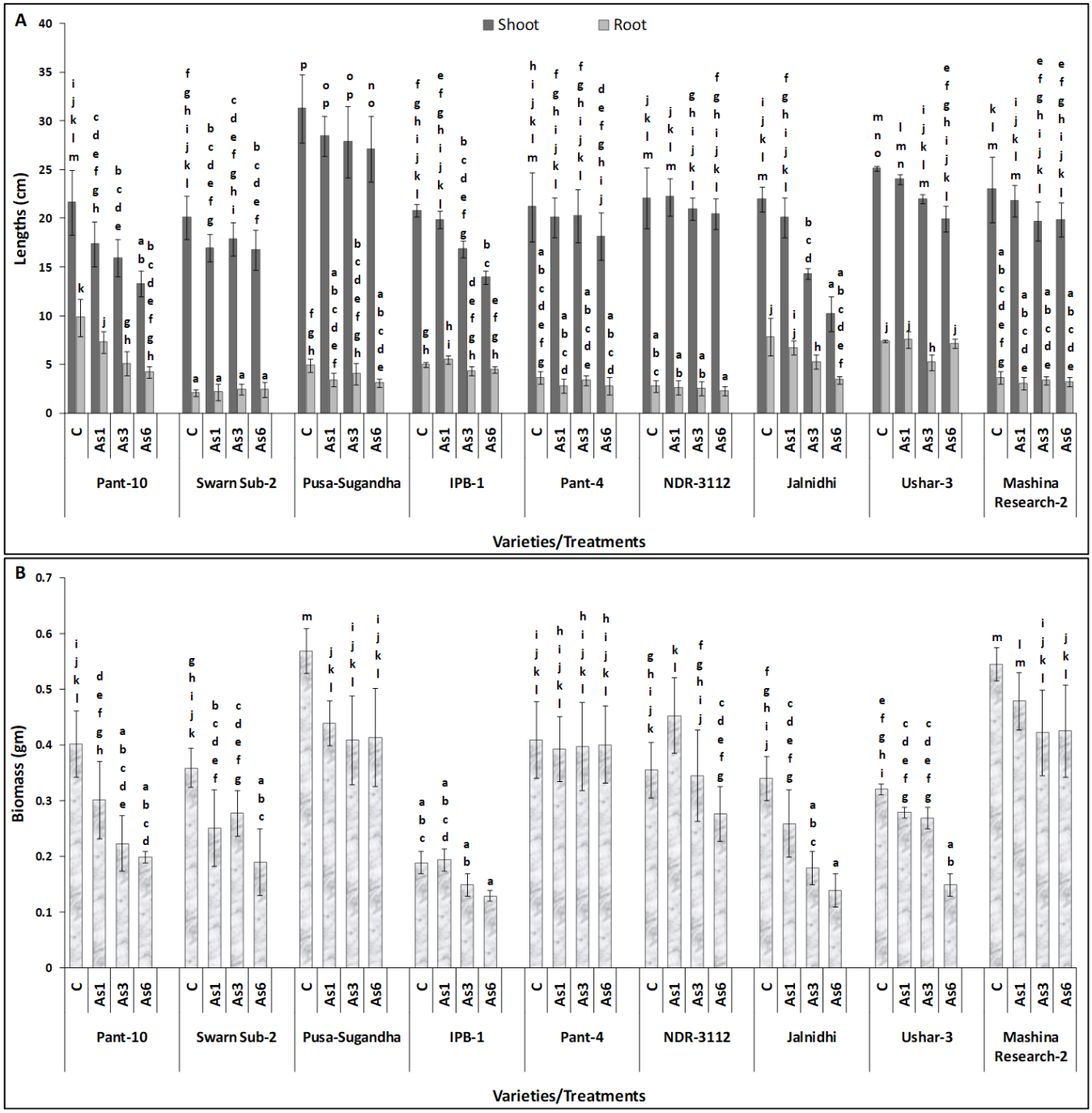

Fig. 2: Effect on shoot, root lengths (cm) and biomass (g) in nine cultivars of rice seedlings treated with different concentrations of As (1, 3, 6 µg ml^{-1}) after 8d of treatment. All values are mean of four replicates ± SD.

observed relative to As(4). On the contrary, application of Se with As(2) i.e., As(2)+Se(2) and As(2)+Se(4) resulted in non-significant change in the growth of treated seedlings, as compared to As(2), except in As(2)+Se(4) where the root length decreased significantly. Whereas, co-treatment of Se(2) and Se(4) with As(4) caused non-significant increase in growth parameters as compared to As(4). However, the addition of P(3) to a combination of Se(2) and As(2), resulted in non-significant decrease in shoot and root lengths of the plants. Whereas, increase in biomass of the treated plants was not inhibited compared to both As(2) and As(2)+Se(2). Similarly, addition of Se(2) and P(3) to both sets of As(4) i.e., As(4)+Se(2)+P(3) and As(4)+Se(4)+P(3) have shown non-significant increase in growth, in comparison to As(4). Shoot lengths (21.75% and 21.43%) and biomass (30.76% and 53.84%) of the plants increased significantly by addition of higher rate of P [P(6)] to As(4)+Se(2) and As(4)+Se(4), whereas, root lengths were affected non-significantly when compared with As(4). Among all sets of treatments, maximum increase in shoot length (19.67%) and in biomass (53.84%) was observed in As(4)+Se(4)+P(6) over As(4). Quite contrarily, treatment comprising of P(3) and P(6) with As(4) (without Se) have resulted into significant decrease in root and shoot lengths and non-significant decrease in biomass, except in As(4)+P(6) (Fig. 3).

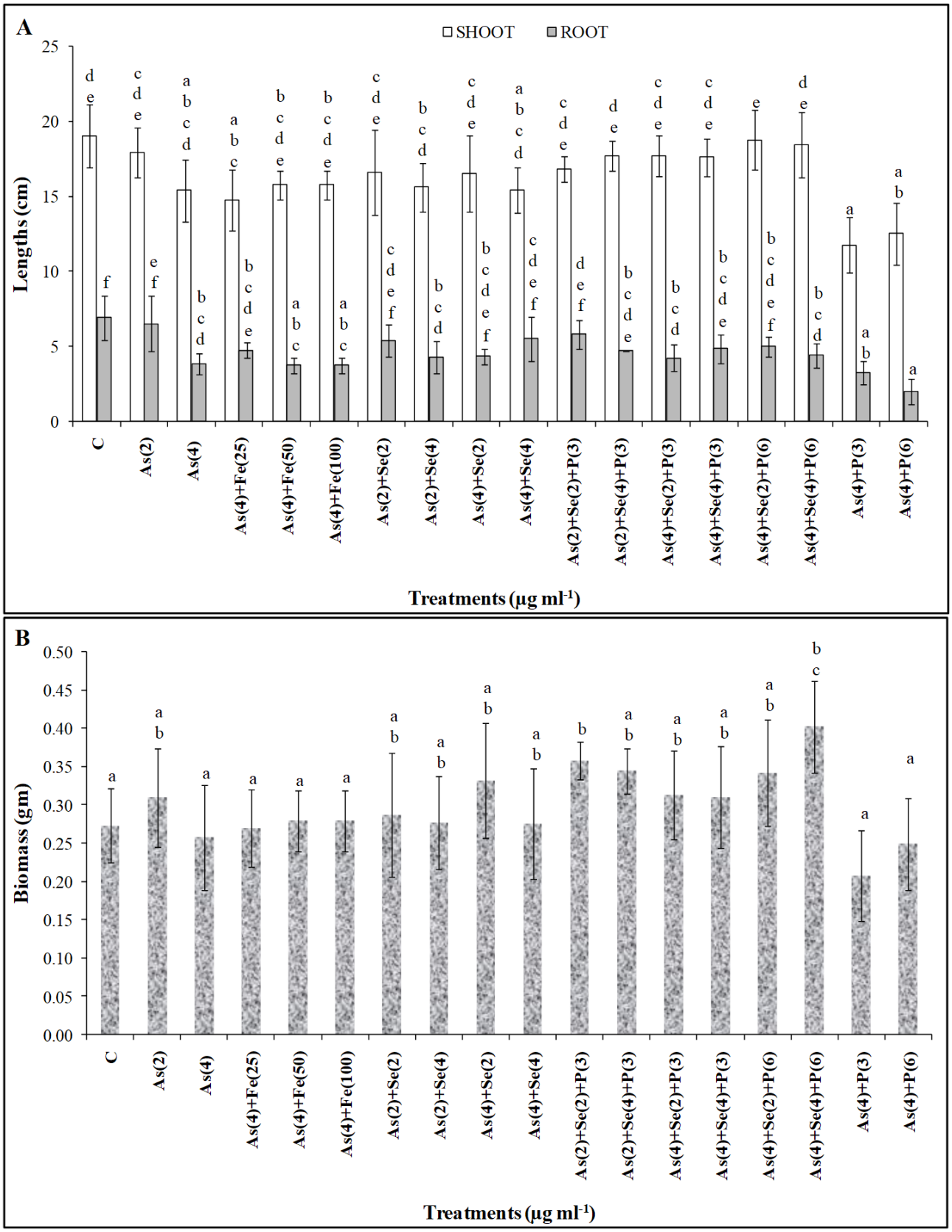

Fig. 3: Effect on (A) shoot, root lengths (cm) and (B) biomass (gm) in the plants treated with As alone and by the addition of different concentrations of Fe, Se and P after 8d. Error bars represent ± SD of four replicates. Horizontal bars indicated by same letters are not significantly different (DMRT, $p < 0.05$).

Table 2: Effect on shoot, root lengths (cm), biomass (g) and accumulation (µg g^{-1} dw) in nine cultivars of rice seedlings treated with different concentrations of As (1, 3, 6 µg ml^{-1}) after 8d of treatment. All values are mean of four replicates ± SD.

	Arsenic accumulation (µg ml^{-1})																	
	Pant-10		*Swarn Sub-2*		*Pusa-Sugandha*		*IPB-1*		*Pant-4*		*NDR-3112*		*Jalnidhi*		*Ushar-3*		*Mashina Research-2*	
Treatments	*Root*	*Shoot*	*Root*	*Shoot*	*Root*	*Shoot*	*Root*	*Shoot*	*Root*	*Shoot*	*Root*	*Shoot*	*Root*	*Shoot*	*Root*	*Shoot*	*Root*	*Shoot*
C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
As(1)	192.0 ± 29.3e	10.0 ± 1.6bc	37.8 ± 18.1a	9.0 ± 2.2ab	439.9 ± 7.3h	13.6 ± 3.1cd	181.7 ± 35.4de	17.8 ± 2.1d	111.3 ± 9.0c	6.6 ± 0.7ab	20.9 ± 5.0a	5.1 ± 0.1a	301.5 ± 0.0f	9.3 ± 0.0b	132.1 ± 16.9cd	23.2 ± 1.1e	98.0 ± 0.0bc	17.0 ± 2.9d
As(3)	604.8 ± 101.9i	30.4 ± 3.8f	181.5 ± 27.7de	14.9 ± 0.0d	480.6 ± 25.6h	16.0 ± 0.0d	202.1 ± 0.0e	23.0 ± 1.9e	299.8 ± 13.4f	25.2 ± 1.5e	53.1 ± 9.2ab	6.2 ± 1.9ab	330.8 ± 0.0fg	32.6 ± 0.0fg	199.4 ± 13.7e	33.2 ± 2.6fg	222.6 ± 0.7e	32.3 ± 2.6fg
As(6)	784.7 ± 74.7j	60.3 ± 2.2k	284.0 ± 14.8e	22.7 ± 4.6e	565.9 ± 24.6i	17.4 ± 2.4d	173.0 ± 0.0de	42.8 ± 3.1j	178.7 ± 6.2de	41.6 ± 3.6ij	118.1 ± 2.4c	14.2 ± 2.8d	369.2 ± 0.0g	45.3 ± 0.0j	190.9 ± 11.1e	38.1 ± 3.6hi	224.3 ± 7.7e	35.7 ± 2.3gh

Table 3: Accumulation (µg g^{-1} dw) of As in the roots and shoots of cv. Pant-10 in As(III) in different concentrations of Fe, Se and P and essential elements As(III) and As(III)+Fe treated plants after 8d. All values are mean of four replicates ± SD. Value with same letters in the same column are not significantly different (DMRT) (Mean± SD; n=4).

Treatments	*As accumulation*		*Se accumulation*					
	Shoots	*Roots*	*Shoots*	*Roots*	*Cu*	*Zn*	*Mn*	*Fe*
C	-	-	-	-	8.6±1.5^{a}	25.9±2.7^{ab}	45.1±10.8^{a}	307.0±50.4^{a}
As(2)	24.8±0.6^{d}	195.7±3.3^{d}	-	-	13.9±3.4^{b}	23.221.8^{a}	58.2±7.4^{a}	267.2±21.7^{a}
As(4)	60.3±2.2^{f}	432.8±50.6^{gh}	-	-	14.2±2.6^{b}	23.2±5.5^{a}	59.4±14.6^{ab}	259.5±80.5^{a}
As(4)+Fe(25)	36.6±3.2^{e}	528.3±1.6^{i}	-	-	14.7±1.2^{b}	25.5±2.6^{ab}	78.2±9.67^{b}	343.2±44.6^{a}
As(4)+Fe(50)	26.7±1.1^{d}	469.9±42.8^{h}	-	-	18.3±1.5^{c}	31.0±3.2^{b}	78.6±7.7^{b}	1036.8±78.7^{b}
As(4)+Fe(100)	6.9±2.0^{a}	414.8±11.1^{g}	-	-	20.5±2.3^{c}	28.3±2.7^{ab}	126.7±11.6^{c}	2141.6±440.4^{c}
As(2)+ Se(2)	16.7±3.8^{bc}	56.2±6.7^{ab}	22.9±7.9^{a}	102.9±3.2^{a}	-	-	-	-
As(2)+Se(4)	11.9±5.2^{ab}	45.0±5.1^{a}	21.6±8.9^{a}	174.8±9.1^{c}	-	-	-	-
As(4)+Se(2)	24.6±3.6^{d}	134.4±1.1^{c}	24.8±9.4^{a}	141.2±0.4^{b}	-	-	-	-
As(4)+Se(4)	22.7±5.7^{cd}	69.6±3.8^{ab}	24.5±4.6^{a}	129.9±6.1^{b}	-	-	-	-
As(2)+Se(2)+P(3)	11.1±0.7^{ab}	109.6±13.6^{bc}	24.4±15.2^{a}	124.8±1.6^{b}	-	-	-	-
As(2)+Se(4)+P(3)	10.5±1.8^{ab}	56.2±4.5^{ab}	22.5±7.7^{a}	181.5±4.6^{c}	-	-	-	-
As(4) +Se(2)+P(3)	34.8±3.1^{e}	87.3±5.1^{abc}	22.5±11.4^{a}	94.1±6.7^{a}	-	-	-	-
As(4)+Se(4)+P(3)	23.3±2.3^{d}	69.1±8.6^{ab}	37.5±5.0^{a}	130.2±14.0^{b}	-	-	-	-
As(4)+Se(2)+P(6)	22.4±2.1^{cd}	116.0±15.3^{bc}	20.6±6.1^{a}	90.0±11.1^{a}	-	-	-	-
As(4)+Se(4)+P(6)	16.1±2.9^{b}	69.7±7.6^{ab}	20.8±9.5^{a}	129.4±15.0^{b}	-	-	-	-
As(4)+P(3)	73.3±6.7^{g}	330.1±82.0^{f}	-	-	-	-	-	-
As(4)+P(6)	80.7±2.7^{h}	263.5±5.2^{e}	-	-	-	-	-	-

Experiment I and II: Accumulation of iAs and Se in different treatments

With increase in iAs concentration As(2) to As(4), the uptake of iAs increased significantly in both the parts of the plant, relative to their respective values in C. However, the addition of Fe to As(4) decreased the iAs translocation to the shoots significantly, over As(4), with maximum decrease (88.63%) in As(4)+Fe(100) shoots (Table 3). Significant decrease in iAs uptake was also observed in the shoots of As(2)+Se(2) [32.83%] and As(2)+Se(4) [52.07%] as well as in its roots [71.31% and 77.03%, respectively] over As(2). However, with increasing Se concentration from As(2)+Se(2) to As(2)+Se(4), iAs uptake decreased in the shoots which were not statistically significant. Likewise, decrease in iAs uptake was also observed in both the parts of the seedlings treated with Se and higher rate of As i.e., As(4)+Se(2) and As(4)+Se(4), as compared to As(4). Further addition of P to As+Se treatments, did not show significant reduction in shoot As uptake, in comparison to their respective As+Se values, except in As(4)+Se(4)+P(6) where it decreased significantly by 29% as compared to As(4)+Se(4) and 73.33% compared to As(4). In contrast, the seedlings co-treated with higher and lower rates of P exhibited significant increase in shoot iAs uptake i.e., As(4)+P(3) [21.67%] and As(4)+P(6) [33.89%], over As(4).

In all sets of Se treatments with As(2) and As(4), no significant difference was observed in shoot Se uptake (Table 3). In roots, the uptake of Se increased with increase in Se concentrations, as evident from the higher uptake in As(2)+Se(4) and As(4)+Se(4) as compared to As(2)+Se(2) and As(4)+Se(2), respectively.

Overall, results showed that the application of Fe was more efficient to reduce As accumulation than Se and P combinations against As(III) treatments.

Experiment II: Toxicity and defence mechanism

The oxidative stress evaluated in terms of increased TBARS and H_2O_2 levels with the As+Fe [Fig. 4a(A)] was higher than the iAs treated plants. Within iAs treated plants, the level of MDA increased non-significantly with increase in iAs concentration, at both the exposure periods, however, with addition of Fe the levels increased significantly in all the sets of treatments at 4d, in comparison to C. Maximum increase was observed in As(4)+Fe(100) both after 4d (107.42%) and after 8d (33.84%), as compared to As(4). Similarly, significant increase in H_2O_2 content was observed with the As(4)+Fe(100) treated plants (53.85%), as compared to As(4) after 8d [Fig. 4a(B)]. While no significant change in the AO activity was observed after 4d, however, after 8d its activity in As(4)+Fe(100) significantly decreased (25.15%). SOD activity in all the sets of the treatments, increased significantly in both after 4d and 8d relative to C, except in As(4) [Fig. 4a(C)]. Compared to As(4) at 8d, the SOD activity increased 24.34% significantly with addition of Fe(25) to As(4) and 35.62% with addition of Fe(50) to As(4), however, the addition of higher rate of Fe i.e., Fe(100) have shown no further enhancement in its activity. APX activity increased with exposure to iAs concentration in dose dependent manner with significant increase in As(4) (28.74%) after 4d, whereas, after 8d it increased significantly in As(4)+Fe(25) (69.62%) and with As(4)+Fe(50) (69.22%) [Fig. 4a(D)]. CAT activity between the treatments have shown non-significant difference after 8d, as compared to C and As(4), except in As(4)+Fe(50), where significant increase of 27.50% and 14.38% was observed in comparison to both C and As(4), respectively [Fig. 4a(E)]. POD activity in As(2) and As(4) increased progressively after 4d, in contrast, it decreased after 8d, as compared to the C [Fig. 4a(F)]. Comparison between As(4) treated plants with the As(4)+Fe treated plants, the GST activity increased in As(4)+Fe(25) after 4d and 8d by 14.08% and 10.74%, respectively, whereas with higher rates of Fe i.e., in As(4)+Fe(50) and As(4)+Fe(100), no substantial increase was observed. Gradual decline in the GST activity was observed in a dose dependent manner with increase in iAs concentration after 4d, however, upon addition of Fe(25) there was a recovery of 15.43%, which gradually declined with increase in Fe concentration i.e., As+Fe(50) and As+Fe(100) [Fig. 4a(G)]. The GR activity did not show any significant change at both the exposure periods [Fig. 4a(H)]. After 4d, the GST activity lowered significantly in As(4), however no conclusive inference could be drawn.

Analysis of non-denaturing PAGE activities of the various antioxidant enzymes after 8d (Fig. 4B) showed two isoforms of GPX (A1 and A2) in As+Fe treated plants where the activity was intense relative to C and As(4). Out of the four isoforms of SOD observed, three isoforms i.e., C1, C2 and C3 exhibited higher activity in As+Fe treated shoots, however, the band C4 was invisible in As+Fe treatments. In APX, band B2 was invisible in As+Fe treatment, whereas, the intensity of B3 increased relative to C and As(4) after 8d.

Experiment II: As and Fe treatment and subsequent effect on the essential elements

Among the four essential elements studied in As and As+Fe treated plants, the level of Cu increased in all the treatments, as compared to that in C, Mn level increased in iAs treated plants and significantly in As+Fe treated plants, where a significant increase of 113.15% was observed in As(4)+Fe(100), against As(4) (Table 3). The level of Fe increased in a dose dependent manner with increase in As concentration. The level increased 112.12% in As(4)+Fe(100) as compared to As(4). However, all treatments of Fe with As have shown non-significant difference in Zn levels.

Discussion

The reduction in the growth parameters of the rice seedlings against various treatments is congruent with similar results from earlier studies involving different plants treated with iAs (Malik *et al.,* 2012; Farooq *et al.,* 2016; Abbas *et al.,* 2018). Deng *et al.* (2010) reported that the addition of Fe (30 mg L^{-1}) increased adsorption of both As(III) and As(V) on the root surface which minimized the As uptake into the roots and subsequent translocation to the shoots. A number of studies have shown that Fe-plaque could act as a barrier to the uptake of other toxic metals in rice. It is now proven that Fe is deposited on the rice root surface of aquatic plants as Fe-(oxyhydr) oxides (Fe plaques) which reduces the overall root surface area available for As uptake and some amount of As gets adsorbed on the Fe-plaques (Syu *et al.,* 2013; Hu *et al.,* 2015). In agreement with these studies, in the present study, inhibition of iAs uptake was more pronounced in As+Fe treated plants which could be due to the formation of Fe plaque on the root surface which acted as a barrier towards uptake of iAs.

On the contrary, Liu *et al.* (2005) and Chen *et al.* (2005) observed that the As levels in the shoots of rice plants treated with As(III) for two weeks, were higher in the Fe-plaque induced plant than those on the non-Fe plaque plants, and the reverse was true with As(V). The uptake and translocation of the As(III) into the shoots of As+Fe treated plants could be due the reason described by Meharg and Jardine (2003) where As(III) adsorbed on Fe plaque are in the form of H_3AsO3^0 which are transported into rice roots via root aquaporins.

On the contrary, the uptake of iAs in the shoot was higher in the presence of P. Lou-Hing *et al.* (2011) reported low phosphate (<50 µM) did not affect iAs uptake, whereas, high phosphate (≥50 µM)

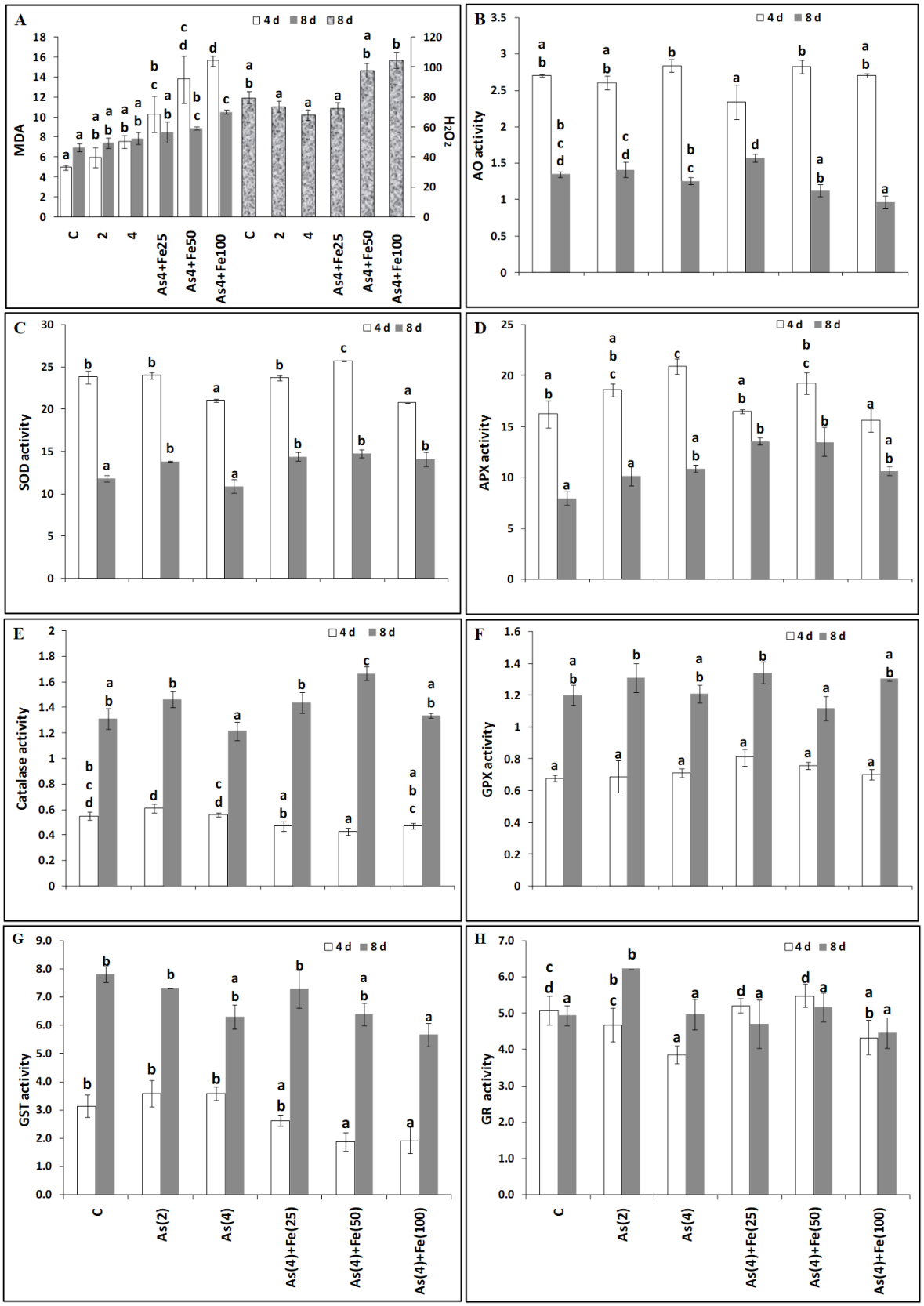

Fig. 4: (A) Effect on (A) MDA content (mmol g^{-1} fw), H_2O_2 (µg g^{-1}fw), (B) AO (U mg^{-1} protein), (C) SOD (U mg^{-1} protein), (D) APX (µmol min^{-1} mg^{-1} protein), (E) CAT (mmol min^{-1} mg^{-1} protein), (F) POD (mmol min^{-1} mg^{-1} protein), (G) GST content (mmol mg^{-1} protein), (H) GR (U mg^{-1} protein) in the shoots of the plants grown in As and As+Fe treatment after 8d. All the values are means of four replicates ±SD. Horizontal bars indicated by the same letters are not significantly different (DMRT, $p < 0.05$).

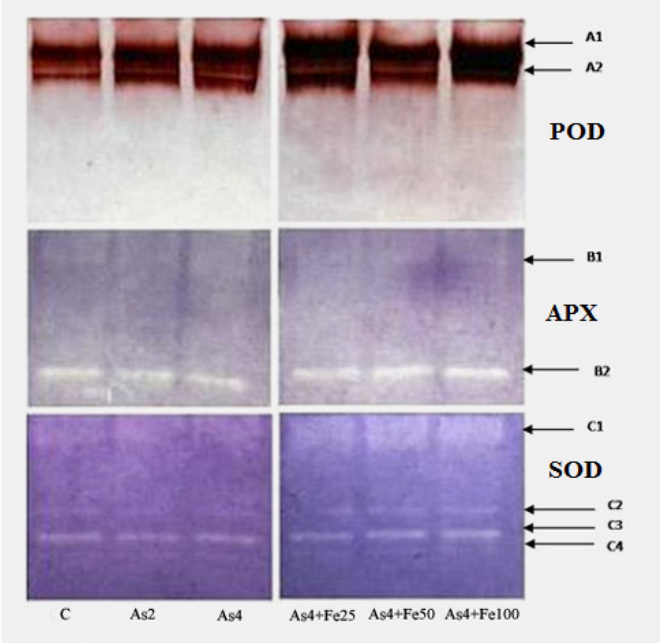

Fig. 4b: Native PAGE activity of different isoform of POD, APX and SOD in the shoot extract of rice seedlings treated with different concentrations of As and Fe.

treatment provided protection to both As(V) and As(III) (13.3 µM) in rice plants. Lu *et al.* (2010) suggested alteration in shoot P status as promising criteria for breeding rice with reduced grain iAs. Also, simultaneous addition of Se and P have shown antagonistic roles in As translocation to the shoots which corroborate with the earlier finding of Kumar *et al.* (2013). However, overall results indicate that the application of Fe efficiently reduced the iAs accumulation than Se and P combinations, although inflicted higher toxicity. The higher reduction of iAs accumulation with Fe application than against Se+P combination, possibly could be owing to Fe plaque formation on rice root. The Fe is well known to reduce the iAs accumulation, and also reduces Se accumulation through formation of Fe plaque on root surface (Zhou *et al.*, 2007). The reduced Se accumulation may also reduce the efficiency of rice plants abate iAs uptake. Iron not only restricts the accumulation of heavy metals but is also essential for the plant metabolism, while, higher accumulation of Se can induce phyto-toxicity (Kumar *et al.*, 2013; Li *et al.*, 2019).

Selenium has been reported by Malik *et al.* (2012) to inhibit the iAs induced toxicity in *Phaseolus aureus* when simultaneously treated with 10µM As and lower rate of Se (2.5 µM), avowed with this the application of lower rates Se with iAs resulted in 22% recovery of biomass, whereas, no significant abatement in the iAs uptake was observed. In contrary, application of Se at higher rate (5µM) significantly reduced iAs uptake in the plant. In concurrence to our findings, Feng *et al.* (2009) also observed that the uptake of iAs in Chinese brake fern was suppressed by the addition of Se, indicating the antagonistic effect of Se on As uptake. However, in the present study, no significant difference in uptake of Se was observed in any combinations of Se treatments. These authors further reported that increasing the amount of As stimulated the uptake of Se at >2.5 mg L^{-1}, however, at higher rates of Se, As suppressed the uptake of Se. All these results indicate that there is a competitive inhibition of iAs uptake in the plants by the addition of Se, which is transported in the plants by NIP transporter localized at distal side of both exodermis and endodermis of rice roots (Li *et al.,* 2015). This may result into reduction in iAs uptake, in a competitive manner, eventually lowering the inhibition in growth. Similarly, Afton *et al.* (2009) reported simultaneous decrease in Se (44.4%) and As (25.0%) in the roots of *Chlorophytum comosum*, when applied in combination of Se(IV) and As(III) compared to their single treatments. Also, the uptake of Se by the roots of the seedlings increased with application of P and further translocation to the shoots. This is in agreement with Liu *et al.* (2004a,b) where they observed that increase in selenite supply from 2 to 10 µmol l^{-1} resulted in a 2.1-3.5-fold increase in the Se concentrations in rice shoots and 2.4-4.5 fold increase in roots at three P levels. Studies on interaction between As and Se have shown that chronic and acute Se toxicities are alleviated by the administration of sub-acute doses of As(III) and certain other arsenicals in animal systems, which leads to excretion of Se into the gastrointestinal tract (Sun *et al.,* 2014). Recently, the molecular basis of *in-vivo* antagonism between As(III) and Se(IV) has been demonstrated in animal system through the formation of a As-Se compound: seleno-bis (S-glutathionyl) arsinium ion, $[(GS)_2AsSe]^-$ in erythrocytes, which is subsequently excreted in bile of the animal (Prince *et al.* 2007). Similar, compound or metabolism had not been reported in plants. However, in plants Se plays a pivotal role in iAs detoxification by being a part of several seleno-proteins i.e., glutathione peroxidase and thioredoxin reductases (Li *et al.*, 2008).

There have been several studies in recent past involving alleviation of iAs toxicity in rice through essential elements such as Fe, Se and P. Lou-Hing *et al.* (2011) studied the effect of P on iAs uptake, Syu *et al.* (2013) Fe on iAs uptake, Liu *et al.* (2004a) on the combined effect of Fe and P on As uptake in rice. Recently, Kumar *et al.* (2013) has reported that the co-application of Se(IV) and P can reduce iAs uptake in rice seedlings. In concordance with the earlier study, the present study again reports a significant reduction in iAs translocation to the shoots of As(2)+Se(2)+P(3) treated rice seedlings and concomitant increase in growth parameters relative to As(2). In case of As(4)+Se(2), As(4)+Se(2)+P(6), As(4)+Se(4)+P(6) treatments have shown significant decrease in iAs translocation to the shoot along with better growth relative to their respective iAs and As+Se treatments. Thus, the addition of P along with Se can be considered as a suitable option for restricting uptake of iAs in the shoots because both P and Se are fertilizer and micronutrient, respectively, contributing towards the growth of the plant. However, application of the higher rate of Se (4 µg ml^{-1}) to the As has not shown encouraging results in terms of growth.

The treatment of As with P in both the rate i.e., 3 and 6 µg ml^{-1} did not reduce the As(III) accumulation in shoot, as the translocation of As(III) increased from root to shoot. This is in agreement with Lou-Hing *et al.* (2011) and Lihong and Gulian (2009) where they observed that in rice plant roots, iAs was high at low P and low at high P levels. At P concentrations greater than and equal to 50µM, P was able to provide a considerable level of protection, leading almost full protection against As toxicity in two cultivars of rice. Also, the result supports that the iAs supplied as As(III) did not convert to As(V), hence, P could not inhibit iAs uptake.

The rice seedlings under various treatments exhibited, enhanced level of TBARS and H_2O_2 which indirectly indicated the prevalence of ROS under stress conditions, which was more pronounced in As+Fe relative to seedlings treated with iAs. Mishra *et al.* (2011) reported that lipid peroxidation and H_2O_2 levels increased significantly in two cultivars of rice (Malviya-36 and Pant-12) treated with As(III) and the simultaneous increase in antioxidant levels, which serves to mitigate As(III)-induced oxidative damage. The

results of the present study are also in agreement to Mishra *et al.* (2011); where iAs induced toxicity is countered by the antioxidants. The role of Fe in modulation of antioxidants in rice under iAs toxicity has not been reported earlier, as many micronutrients are also speculated to be compromised due to Fe-plaques formation, however, the existing studies deals with restriction of iAs uptake to the rice shoots solely due to Fe plaques (Deng *et al.,* 2010). In As+Fe treated plants, the increase in TBARS and H_2O_2 levels were more pronounced compared to the seedlings treated to iAs alone, which could be due to Fe induced ROS generation through Fenton type reaction, which has coorelation with the higher level of Fe in As+Fe treated plants. However, the increased level of antioxidants in As+Fe plants may serve as important component in mitigating oxidative damage (Awasthi *et al.,* 2017). The higher levels of Mn and Cu in iAs and As+Fe treated shoots, shows that translocation of both Cu and Mn increases with both iAs and As+Fe treatment, with respect to C. In agreement to the variation of observed level of Zn in rice shoots analyzed from three iAs contaminated districts of Bangladesh where variation in the soil level of Zn was the primary reason (Williams *et al.,* 2009). The present study also did not observe any difference in the Zn level in shoots influenced due to the presence of As.

Conclusion

The results demonstrate that the simultaneous application of Se and P could decrease the iAs uptake of the rice shoots. Thus, a suitable concentration of Se and P can be recommended to minimize iAs uptake in rice. Although, the addition of Se alone to the plants has shown significant decrease in iAs translocation to the shoots, while it causes slight toxicity to the seedlings. However, the application of Fe(100 µg ml^{-1}) to As (4 µg ml^{-1}) resulted in minimization of iAs uptake by 89% without affecting the growth of the plant, which indicates that the Fe is essential element than the Se. Among all the antioxidants, the enhanced activity of SOD and APX revels that these enzymes are actively involved in the tolerance mechanism against As(III) toxicity in plants. Overall, study indicates that the application of Fe is great option to reduce the As(III) toxicity than Se and P.

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