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Influence of Arsenate and Phosphate on the Regulation of Growth and TCA Cycle in the Rice (*Oryza sativa* L.) Cultivars IR64 and Nayanmani

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Abstract

The influence of arsenate and phosphate on the growth and respiration of 21 days old seedlings in two cultivars of rice, viz., IR64 and Nayanmani was studied. As arsenate and phosphate are similar in their chemical configuration and the latter is preferentially taken up by the phosphate transporters, it results in a competitive inhibition of arsenate uptake in presence of phosphate. Increasing concentrations of sodium arsenate (25 μM, 50 μM and 100 μM) hindered the growth in both the cultivars, with cv. IR64 being more severely affected than cv. Nayanmani. There was an elevation in the levels of organic acids measured in both the cultivars, accompanied by a reduction in the activities of the dehydrogenases of the TCA cycle, viz., pyruvate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase and malate dedrogenase under arsenic treatment alone. Also, an elevation in the activities of citrate synthase and fumarase enzymes was noticed in both test seedlings with increasing concentrations of arsenic. These alterations were more prominent in cv. IR64 than in cv. Nayanmani. On joint application of phosphate along with arsenate, amelioration of the toxic effects of arsenate was observed to some extent, resulting in an overall revival of respiration leading to improved growth and metabolism.

Keywords

Arsenic, Phosphate, Respiration, Rice, TCA Cycle, Amelioration

1. Introduction

Groundwater arsenic contamination leading to toxicity in plants, animals and humans has become a major concern in the last few decades [1]. In parts of In-

dia and Bangladesh, drinking water can contain more than 50μg/L of arsenic, while the concentration of arsenic may be as high as 400 μg/L in the rice fields [2] owing to the use of arsenic contaminated groundwater for irrigation. Rice, the major staple food crop of half the world's population is particularly efficient in accumulating arsenic from the soil and hence poses a major risk to human health.

Arsenic is present in the environment in various forms; the major biologically important species are arsenite (As III) and arsenate (As V) [3]. Arsenic shows various phytotoxic effects like stunted shoot and root growth, yellowing of leaves [4] along with reduction in the photosynthetic capacity of the cell [5]. This is the result of reaction of pentavalent and trivalent arsenic radicals with the sulfhydryl groups and replacement of phosphate from ATP [6].

The TCA cycle is a series of chemical reactions used by aerobic organisms to generate energy. Till date, very few reports exist on effect of arsenic on respiration in plants, although it has been reported that heavy metals like cadmium, copper and lead affected the respiration in plants considerably. Studies on the cell membrane ultrastructure of tobacco leaves by [7] showed that Cd toxicity altered respiration by damaging the mitochondrion cristae. Moreover, it was found that both copper and lead adversely affected the seed germination and respiratory rates in soyabean and rice respectively [8] [9].

Many experiments have co-related the production of organic acids to abiotic stress tolerance in plants. Functions of organic acids, especially TCA cycle intermediates are manifold, ranging from assisting with nutrient deficiencies, metal tolerance and plant-microbe interactions at the soil-root interphase to being involved in varied biochemical pathways like production of energy and formation of precursors for amino acid biosynthesis [10].

Pyruvate, which is yielded as a result of glycolysis, is decarboxylated by the enzyme pyruvate dehydrogenase (PDH) (EC 1.2.4.1) forming acetyl coenzyme A which then enters the TCA cycle. In the next reaction the enzyme citrate synthase (CS) (EC 2.3.3.1) combines the acetyl group of acetyl-CoA with a four-carbon dicarboxylic acid (oxaloacetate, OAA) to give a six-carbon tricarboxylic acid (citrate) [11]. Other important steps in the cycle are conversion of isocitrate to a-ketoglutarate catalyzed by isocitrate dehydrogenase (ICDH) (E.C. 1.1.1.41), succinate to fumarate catalyzed by succinate dehydrogenase (SDH) (E.C. 1.3.5.1) with the generation of energy in the form of $FADH_2$ and malate to oxaloacetate catalyzed by malate dehydrogenase (MDH) (E.C. 1.1.1.37). Fumarase (E.C. 4.2.1.2.) catalyses the reversible hydration of fumarate to malate, one of the constituent reactions of the tricarboxylic acid (TCA) cycle [12].

Phosphorus (P) is one of the essential elements required for plant growth and is a chemical analogue of arsenic as both the elements belong to group Va of the periodic table. Because of the similarity in their chemical structure, As and P compete for the same uptake carriers in the plants [13]. Studies indicate that addition of P resulted in reduced uptake of As in arsenic tolerant plants like *Holcus lanatus*, *Cystisus striatus* [14] [15] as well as in *Brassica juncea* [16].

In order to understand the alterations due to application of arsenic with or without phosphate on the overall growth and metabolism of rice seedlings it is imperative that we investigate the effects on the key enzymes and the substrates of the respiratory cycle. By joint application of phosphate along with arsenate, we tried to combat the critical problem of groundwater arsenic toxicity in rice. Such studies will help to devise a cost effective and farmer friendly way to overcome the present situation by application of phosphate enriched fertilizers in arsenic contaminated rice fields.

2. Plant Material and Arsenic Treatments

Rice (*Oryza sativa* L.) seeds cv. Nayanmani and cv. IR64 and obtained from the State Rice Research Station, Chinsurah, Hoogly, West Bengal, were surface sterilized with NaOCl (5% W/V) and washed thoroughly. The petridishes (ϕ10 cm) were lined with filter papers contained about 75 - 80 seeds for each treatment. After germination in dark and humid condition for 72 hours at 30˚C ± 2˚C the seedlings were exposed to various concentrations (25 μM, 50 μM, 100 μM) of sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$; Loba-Chemie, India) solution (w/v) with or without 2 mM potassium dihydrogen orthophosphate (KH_2PO_4; Merck, India) solution (w/v). They were grown hydroponically (Hoaglands solution) [17] with various concentrations of sodium arsenate with or without phosphate and was exposed to 16 h photoperiod for eighteen days. The seedlings were harvested after a total of 21 days for the following studies.

2.1. Morphological Studies

The root and shoot lengths of arsenate treated and untreated seedlings were measured from 21 days old seedlings of both the cultivars. Following this, the separated shoot and root samples were washed, weighed and stored in −40˚C for further biochemical assays.

2.2. Preparation of Supernatant for Assay of Organic Acids Content

Extraction of pyruvic acid, citric acid and malic acid were performed in a similar manner. 1 g of shoot and root samples from each of the treatments were homogenised in 5 ml of 0.2 M phosphate buffer (pH 7.4) and centrifuged for 15 minutes at 10,000 rpm (4˚C). The supernatant was then collected, deproteinized using 5% trichloroacetic acid and filtered. The filtrate was used for the assay of the following organic acid contents.

2.3. Determination of Pyruvic Acid

Pyruvic acid estimation was done according to [18]. The reaction mixture comprised of 1.0 ml deproteinized supernatant, 2.0 ml of 0.2 M phosphate buffer, 0.5 ml of 0.02% DNPH (2,4-dinitrophenylhydrazine). The mixture was incubated for 30 minutes at 37˚C then 5.0 ml of 0.8 N NaOH was added followed by another incubation of 10 minutess at room temperature. The mixture without

plant sample was used as blank. The brown coloured product obtained was measured spectrophotometrically at 510 nm. Amount of pyruvic acid present in the samples was calculated from a standard graph using Sodium pyruvate. The quantity of the total pyruvic acid was expressed as $mg \cdot g^{-1}$ fw.

2.4. Determination of Citric Acid

Citric acid was determined following the method as described by [19]. The assay mixture contained 1 ml filtrate, 5 ml of analytical grade acetic anhydride and 1.3 ml of analytical grade pyridine. The mixture was kept in a water bath for 30 min at 32˚C ± 0.5˚C. The blank contained 1.0 ml distilled water instead of the sample. The optical density (OD) was measured at 405 nm in a Hitachi U-2000 spectrophotometer. A standard curve was prepared using known concentrations of citric acid. The quantity of the total citric acid was expressed as $mg \cdot g^{-1}$ fw.

2.5. Determination of Malic Acid

Malic acid estimation was done according to [20]. The reaction mixture contained 0.5 ml of filtrate, 1.0 ml of 1 N HCl, 0.1 ml of 0.1% DNPH and 0.5 ml of 10% $CaCl_2$. The reaction mixture was incubated at room temperature for 30 minutes, followed by addition of 0.3 ml of 5 N NH_4OH and 6 ml of absolute alcohol. Tubes were kept undisturbed at room temperature for 12 h to complete precipitation. After recentrifugation at 5000 rpm for 15 min, the supernatant was poured off. The tubes were dried in an oven at 105˚C for 15 minutes to remove the moisture. To the dried pellets, 3.0 ml of 0.08% orcinol esulphuric acid mixture was added, the contents were mixed thoroughly with a glass rod and the tubes were heated to 100˚C for 10 min. The mixtures were cooled under the tap water and diluted to 10 ml with concentrated H_2SO_4. The blue fluorescence produced was measured using a Hitachi-650-40 spectrofluorometer against blank. The amount of total malic acid was expressed as $mg \cdot g^{-1}$ fw.

3. Determination of Activities TCA Cycle Enzymes

3.1. Assay of Pyruvate Dehydrogenase

Pyruvate dehydrogenase (E.C. 1.2.4.1) assay was done following the method of [21]. The plant samples were homogenised in 1.5 ml of 50 mM Tris HCl buffer (pH 7.8) containing 0.7 M sucrose, 57 mM β-mercaptoethanol, 2 mM EDTA and 0.5% (w/v) BSA and centrifuged at 10,000 rpm for 20 min at 4˚C. A The assay mixture contained 0.2 ml of the supernatant, 0.2 ml of 50mMTris HCl (pH 8.0) and 0.1 ml each of 5 mM$MgCl_2$, 0.12 mM CoA, 2.6 mM cysteine HCl and 1.5 mM pyruvate making a total volume 1.0 ml . The OD of reaction mixture was measured in a Hitachi U-2000 spectrophotometer with respect to a blank at 340 nm. Then 0.2 ml of 1.4 mM NAD was added to the reaction mixture, mixed well and the absorption was further noted for every 60 s for 2 min. An increasing OD showed the amount of NADH produced per minute. A standard curve with known concentrations of NADH was prepared from which the PDC activity was calculated and expressed as mmol NADH formed $min^{-1} \cdot mg^{-1}$ protein.

3.2. Assay of Citrate Synthase

Citrate synthase (EC 2.3.3.1) activity was determined according to the method described by [22]. Samples were homogenised in a buffer containing 0.1mol/L Tris-HCl buffer (pH 8.0), 0.1% (v/v) Triton X-100 (Triton −100), 2% (w/v) PVP (Polyvinyl polypyrrolidone), and 10mmol/L iso-ascorbic acid. The extracts were centrifuged for 5 min at 15,000 rpm at 4˚C and the supernatant was assayed for enzyme activity. The reaction mixture comprised of 0.1 ml 1mM DTNB (5'-Dithiobis-2-Nitrobenzoic Acid), 0.03 ml of 10 mM acetyl CoA and 0.05ml supernatant. Initial absorbance was recorded followed by addition of 0.05 ml 10 mM OAA(oxalo acetate) and final absorbance was recorded at 412 nm in a Hitachi U-2000 spectrophotometer. Enzyme activity was calculated as µmol citric acid decomposed mg^{-1} protein min^{-1}.

3.3. Assay of Isocitrate Dehydrogenase

Isocitrate dehydrogenase (E.C. 1.1.1.41) activity was determined following the method of [23]. Root and shoot samples were homgenized in 1.5 ml of 50 mM Hepes buffer (pH 7.5) containing 10 mM β-mercaptoethanol and 5% polyvinylpolypyrrolidone. The homogenates were centrifuged at 14,500 g for 20 min at 4˚C. The assay mixture contained 40 mM Hepes buffer (pH 8.2), 2 mM sodium isocitrate, 800 mM NAD, 200 mM $MnSO_4$ and 0.1 ml enzyme making a final volume of 0.5 ml. The increase in absorbance of the reaction mixture was measured in a Hitachi U-2000 spectrophotometer at 340 nm for 2 min. The enzyme activity was expressed as mmol NADH formed $min^{-1}{\cdot}mg^{-1}$ protein.

3.4. Assay of Succinate Dehydrogenase

Succinate dehydrogenase (E.C. 1.3.5.1) activity was determined according to [24]. Root and shoot samples were homgenized in 1.5 ml of 4 mMTris HCl (pH 7.5) buffer containing 0.19 M sucrose. The homogenates were centrifuged at 10,000 rpm for 15 min at 4˚C. The assay mixture contained 0.1 ml of distilled water, 0.05 ml each of 0.19 M sucrose, 0.1MTris HCl (pH 7.5), 10 mM sodium azide, 8 mM INT [2-(p-iodophenyl)-3-(pnitrophenyl)-5-(phenyl tetrazolium chloride)], 0.1 ml of 0.5 M sodium succinate and 0.1 ml supernatant. The mixture was incubated at 30˚C in a water bath for 10 min. 95% alcohol was added to each tube, mixed thoroughly and kept in an ice bath for 10 - 15 min. Finally, the mixtures were centrifuged at 8000 rpm at room temperature for 10 min and absorbance was recorded at 458 nm. Basal reduction of INT was determined in control tubes without succinate. The enzyme activity was expressed as mmol INT reduced $min^{-1}{\cdot}mg^{-1}$ protein.

3.5. Assay of Fumarase

Fumarse (E.C. 4.2.1.2.) activity was estimated according to [25]. Plant samples were ground in liquid nitrogen and extracted in a buffer comprising of 10%(v/v) glycerol. 0.25%(w/v) BSA, 0.1%(v/v) TritonX −100, 50 mM HEPES/KOH pH7.5, 10 mM $MgCl_2$, 1mM EDTA, 1 mM EGTA, 1mM benzamidine, 1mM ε amino

caprionic acid, 1mM phenyl methyl sulphonyl fluoride, 10μM leupeptin and 1mM DTT. The assay mixture comprised of 100 mM Tricine /KOH pH 8.0, 0.2 mM acetyl CoA,5 mM phosphate, 5 mM $MgCl_2$, 0.15 mM NAD+, 0.5(v/v) TritonX −100, 50 units malate dehydrogenase and 1 unit ml^{-1} citrate synthase. The reaction was started by addition of fumarate and was terminated with 20μl 0.5 NaOH. The increase in absorbance was measured at 380 nm at 10 seconds for at least 60 seconds.

3.6. Assay of Malate Dehydrogenase

Malate dehydrogenase (E.C. 1.1.1.37) activity was determined according to [26]. 1 g plant sample from each set was homogenized in 3 ml of 50 mMTris HCl (pH 8.0) buffer containing 50 mM $MgCl_2$, 5 mM β-mercaptoethanol and 1 mM EDTA and centrifuged at 10,000 rpm for 20 min at 4˚C. The assay mixture was prepared with 0.5 ml of 5 mM OAA, 0.5 ml of 10 mM $MgCl_2$, 1.3 ml of 0.1 M Tris HCl buffer (pH 7.8) and 0.2 ml of the enzyme extract. The initial absorbance of the reaction mixture was measured in a Hitachi U-2000 spectrophotometer at 340 nm. Then 0.5 ml of 0.4 mM NADH was added and absorption was recorded every 60 s for at least 2 min. The enzyme activity was expressed as mmol NADH oxidized $min^{-1} \cdot mg^{-1}$ protein.

3.7. Protein Estimation

Protein contents were estimated according to [27], using bovine serum albumin (BSA, Sigma) as standard.

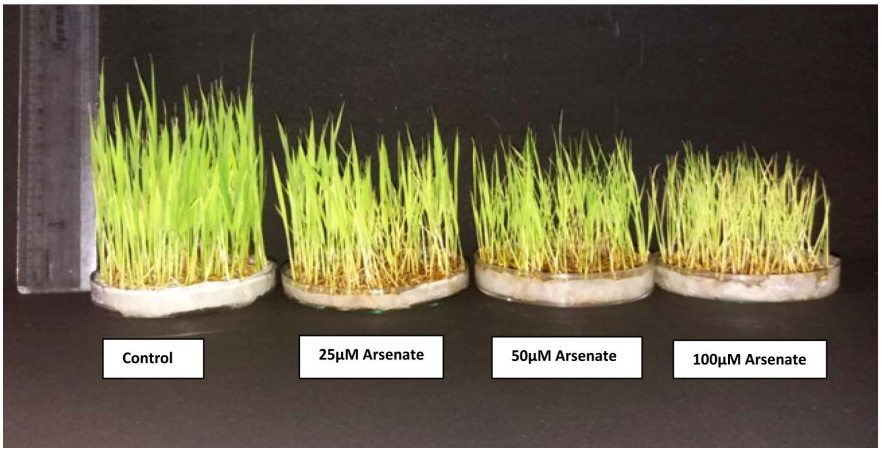
3.8. Statistical Analysis

The experiments were carried out in a completely randomized design (CRD) with 3 replicates; each replication comprised a single petridish containing an average of 70 - 80 seeds. The data and significant differences among mean values were compared by descriptive statistics (±SE) followed by Student's t test. The values of $P \leq 0.05$ were considered as statistically significant.

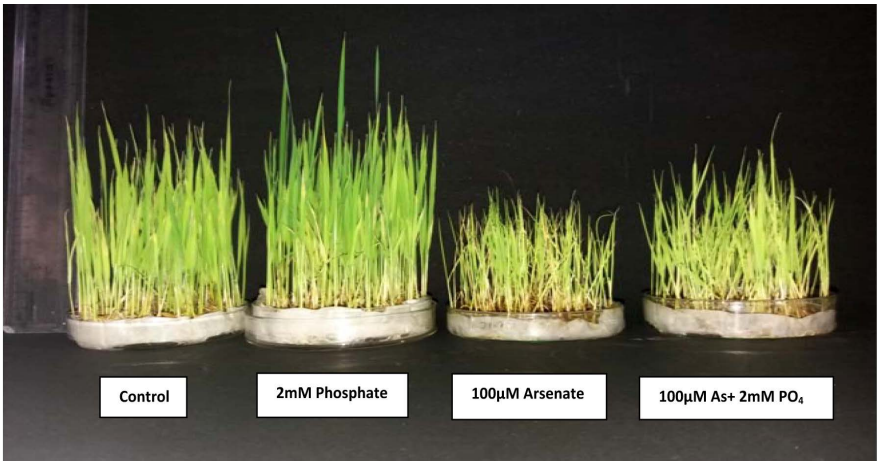
4. Results

4.1. Effect of Arsenate on Growth of 21 Days Old Rice Seedlings

The normal growth of the shoot as well as the root was affected by arsenic toxicity. The shoot and root lengths decreased linearly with increasing concentrations of arsenate in both the test cultivars (**Table 1**), however the reduction in growth was more significant in cv. IR64 than in cv. Nayanmani. In cv. IR64 the reduction in shoot length was 8%, 17% and 51%, while in cv. Nayanmani shoots the decrease was 13%, 20% and 29%. The inhibition in root lengths was 12%, 33%, 52% in cv. IR64 and 9%, 21%, 33% in cv. Nayanmani. On co-application of phosphate along with arsenate, there was considerable revival in growth with cv. IR64 showing 9% and 16% average reduction in shoot and root lengths. In comparison, cv. Nayanmani experienced 7% and 14% inhibition in shoot and root lengths on an average (**Figure 1** and **Figure 2**).



(a)



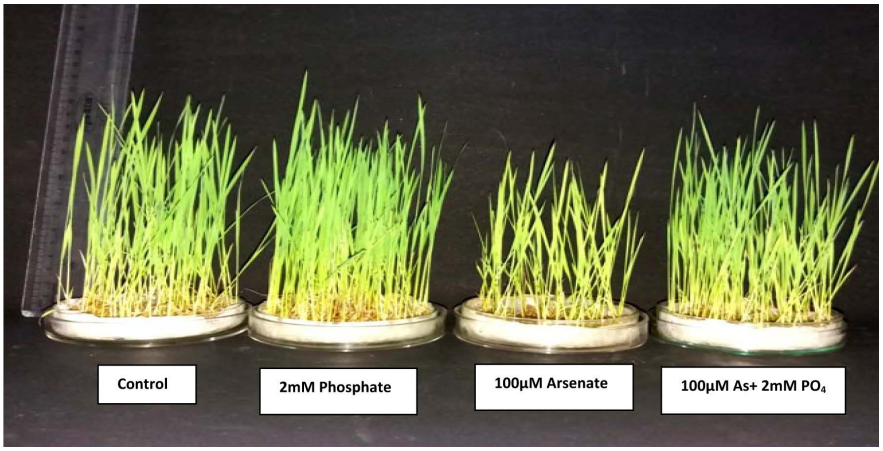
(b)

Figure 1. (a) (b) Effect of arsenate and phosphate applied singly or in combination on the growth of 21 days old rice (cv. IR64) seedlings.

Table 1. Table showing shoot and root lengths of 21 days old rice seedlings.

Treatment	cv. IR64 (length in cm)		cv. Nayanmani (length in cm)	
	Shoot	Root	Shoot	Root
Control	6.95 ± 0.75	10.8 ± 1.22	7.17 ± 0.93	9.57 ± 1.39
Arsenate (µM)				
25	6.42 ± 1.05	9.53 ± 0.45	6.23 ± 0.69	8.67 ± 0.98
50	5.77 ± 0.48*	7.19 ± 0.63*	5.77 ± 0.52	7.57 ± 0.75
100	3.38 ± 0.62*	5.15 ± 0.32*	5.10 ± 0.34*	6.37 ± 0.51*
Phosphate (2 mM)	7.60 ± 0.88	11.0 ± 0.52	7.73 ± 0.81	10.2 ± 0.87
+Arsenate				
25	7.27 ± 0.97	10.2 ± 1.24	7.10 ± 0.46	9.27 ± 0.98
50	6.57 ± 0.23*	9.38 ± 0.89	6.73 ± 0.64	8.43 ± 0.34
100	5.73 ± 1.11	7.57 ± 0.5*	6.17 ± 0.23*	6.90 ± 0.29*

(a)



(b)

Figure 2. (a) (b) Effect of arsenate and phosphate applied singly or in combination on the growth of 21 days old rice (cv. Nayanmani) seedlings.

4.2. Effect of Arsenate on the Intermediates of Krebs' Cycle

The organic acids namely pyruvate, citrate and malate were measured in both the root and the shoot samples of 21 days old rice seedlings of cultivars IR64 and Nayanmani under various concentrations of arsenate (As) treatment. The levels of organic acids were generally higher in the roots than the shoots in both the cultivars under As stress with cv. IR64 (Table 2) experiencing more elevation in comparison to cv. Nayanamani (Table 3).

Root and shoot of cv. IR64 showed 51% and 45% increment in pyruvate levels respectively while cv. Nayanmani experienced 36% and 22% elevation in pyruvic acid contents in its root and shoot on an average respectively under increasing arsenic treatment. Citrate levels also escalated under various concentrations of As stress with cv. IR64 elevation of showing 36%, 77%, 103% in root and 24%, 67%, 114% in shoot under 25 µM, 500 µM and 100 µM As treatments respectively. Cv. Nayanmani in comparison underwent 51%, 89%, 101% and 19%, 67% and 85% elevation in citric acid contents in the root and shoot respectively. Malic acid content in cv. IR64 showed 97% and 212% increment on an average in the root and shoot respectively by arsenic treatment, while cv. Nayanmani

Table 2. Table showing the levels of organic acids content in 21 days old cv. IR64 seedling.

Treatment	Pyruvic acid content (mg/g fw)		Citric acid content (mg/g fw)		Malic acid content (mg/g fw)	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	3.2 ± 0.15	3.5 ± 0.25	0.58 ± 0.09	2.15 ± 0.34	1.29 ± 0.09	2.10 ± 0.02
Arsenate(μM)						
25	3.9 ± 0.90	4.0 ± 0.18	0.72 ± 0.02	2.93 ± 0.21	3.62 ± 0.15*	3.65 ± 0.40*
50	4.3 ± 0.05*	5.5 ± 0.70	0.97 ± 0.14	3.81 ± 0.15*	4.15 ± 0.52*	4.18 ± 0.01*
100	5.7 ± 0.68*	6.4 ± 0.85*	1.24 ± 0.22*	4.36 ± 0.58*	4.32 ± 0.27*	4.59 ± 0.39*
Phosphate(2mM)	3.0 ± 0.28	3.4 ± 0.16	0.45 ± 0.03	2.02 ± 0.29	1.30 ± 0.16	2.02 ± 0.13
+Arsenate						
25	3.3 ± 0.53	4.0 ± 0.40	0.55 ± 0.07	2.09 ± 0.16	1.87 ± 0.31	2.53 ± 0.19
50	3.5 ± 0.45	4.3 ± 0.29	0.61 ± 0.10	2.51 ± 0.24	2.74 ± 0.12*	3.35 ± 0.07*
100	4.6 ± 0.27*	5.9 ± 0.50*	0.89 ± 0.09	3.17 ± 0.41	3.06 ± 0.05*	3.99 ± 0.56*

Table 3. Table showing the levels of organic acids content in 21 days old cv. Nayanmani seedlings.

Treatment	Pyruvic acid content (mg/g fw)		Citric acid content (mg/g fw)		Malic acid content (mg/g fw)	
	Shoot	Root	Shoot	Root	Shoot	Root
Control	2.8 ± 0.49	2.8 ± 0.21	1.60 ± 0.12	1.09 ± 0.21	4.61 ± 0.06	3.78 ± 0.03
Arsenate(μM)						
25	2.8 ± 0.52	3.4 ± 0.17	2.19 ± 0.17*	1.65 ± 0.28	5.75 ± 0.22*	4.92 ± 0.66
50	3.4 ± 0.45	3.8 ± 0.10	2.68 ± 0.20*	2.06 ± 0.22*	6.15 ± 0.75	5.65 ± 0.14*
100	4.1 ± 0.31	4.2 ± 0.09*	2.96 ± 0.28*	2.20 ± 0.16*	6.09 ± 0.39*	5.81 ± 0.49*
Phosphate(2mM)	2.6 ± 0.35	3..4 ± 0.22	1.08 ± 0.40	1.08 ± 0.05	4.22 ± 0.17	3.53 ± 0.07*
+Arsenate						
25	2.8 ± 0.43	2.8 ± 0.07	1.73 ± 0.37	1.08 ± 0.21	4.50 ± 0.29	3.21 ± 0.33
50	3.2 ± 0.26	3.5 ± 0.23	1.80 ± 0.32	1.10 ± 0.26	5.31 ± 0.11*	4.95 ± 0.41*
100	3.8 ± 0.18	4.2 ± 0.12*	1.88 ± 0.28	1.64 ± 0.14	5.39 ± 0.59	5.50 ± 0.58*

showed comparatively less increase of about 44% and 30% on an average in the root and shoot respectively.

Application of 2 mM phosphate along with arsenate in all the cases showed alleviation of arsenic toxicity to different levels resulting in reduced secretion of the organic acids. Pyruvate, citrate and malate contents under joint treatment of arsenate and phosphate showed 17%, 13%, 17% average elevation in Nayanmani shoot and 25%, 17%, 21% average increase in Nayanmani root respectively. In comparison cv. IR64 shoot experienced 18%, 18%, 98% average increase in pyruvate, citrate and malate contents, while cv. IR64 roots showed 35%, 21% and 57% average increase in the concentrations of the above mentioned organic ac-

ids with respect to arsenic treatment alone.

4.3. Effect on the Activities of Krebs Cycle Enzymes

4.3.1. Pyruvate Dehydrogenase Activity

Activity of pyruvate dehydrogenase (PDH) enzyme declined in both the cultivars to varying extent on treatment with increasing doses of sodium arsenate. In cv. Nayanmani, the reduction in PDH activity was about 49% in root followed by 23% reduction in the shoot of the same on an average. The cv. IR64 experienced around 50% and 39% decrease in PDH activity in the root and shoot respectively under As stress. On application of phosphate along with arsenate PDH activity revived to some extent in both the cultivars with cv. Nayanmani showing 12% decline each in the root and shoot, while cv. IR64 experienced 23% and 22% average reduction in the root and the shoot respectively (**Figure 3**).

4.3.2. Citrate Synthase Activity

Roots of cv. IR64 exhibited 23%, 89% and 112% elevation in citrate synthase (CS) activity while the shoots recorded an increment of 45%, 61% and 118% over water control under 25 μM, 50 μM and 100 μM As treatment respectively. In cv. Nayanmani root the reduction in enzyme activity was 15%, 20% and 58% while in shoot the average increase in CS activity was 29% over control under same concentrations of arsenate treatment. Co-application of phosphate along with arsenate showed significant reduction in the activity of CS enzyme with cv. IR64 recording 23% and 25% average elevation in the root and shoot respectively, while the test seedlings cv. Nayanmani showed just 8% and 1% average increase in the root and shoot respectively (**Figure 4**).

4.3.3. Isocitrate Dehydrogenase Activity

The shoot and root of cv. IR64 experienced 21%, 53%, 57% and 26%, 45%, 47%

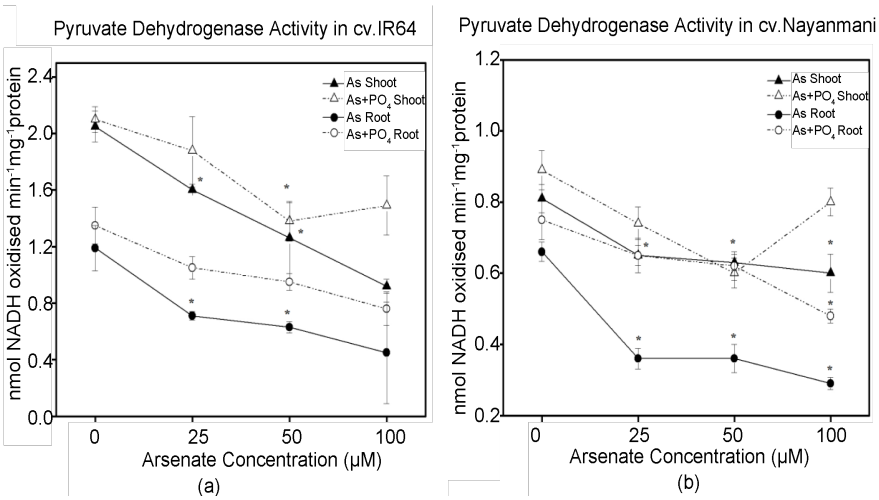


Figure 3. (a) (b) Effect of Arsenate and phosphate applied singly or in combination on the Pyruvate Dehydrogenase (PDH) Activity of 21 days old rice seedlings. The values are means of 3 replicas ± SE. *indicates statistically significant at $P \leq 0.05$ respectively as compared to water control.

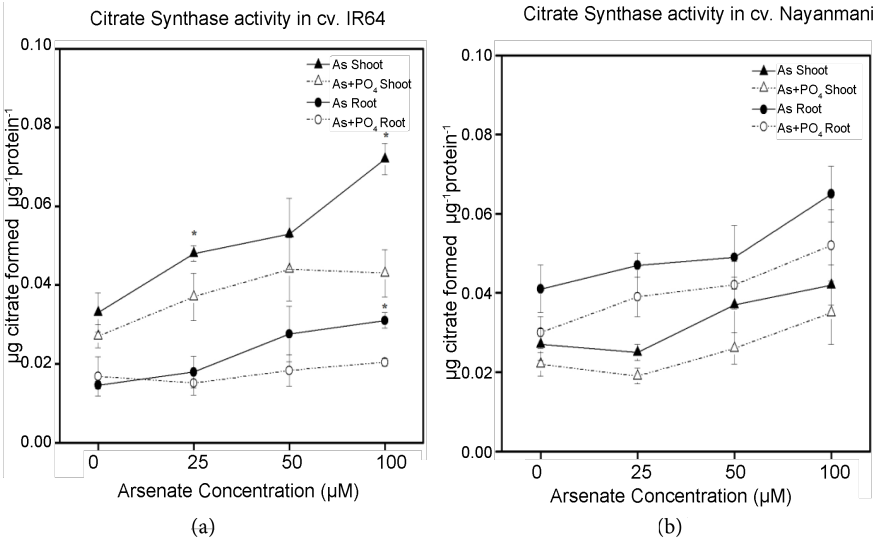


Figure 4. (a) (b) Effect of arsenate and phosphate applied singly or in combination on the citrate synthase (CS) activity of 21 days old rice seedlings. The values are means of 3 replicas ± SE. * indicates statistically significant at $P \leq 0.05$ respectively as compared to water control.

decline in isocitrate dehydrogenase (IDH) activity respectively under 25 µM, 50 µM and 100 µM treatments of sodium arsenate with respect to water control. Addition of 2 mM phosphate along with arsenate resulted in some revival of the enzyme activity in cv. IR64 with the shoot showing average 13% reduction and the root 19% in comparison to water control. Cv. Nayanmani in comparison exhibited less inhibition of IDH activity where the root and shoot displayed 21% and 14% average reduction respectively on arsenate treatment alone, while co-application of phosphate resulted in improved enzyme activity in both the root (10% inhibition) and the shoot (9% inhibition) in comparison to water control (**Figure 5**).

4.3.4. Succinate Dehydrogenase Activity

The enzymatic activity of succinate dehydrogenase (SDH) in cv. IR64 root and shoot reduced by about 42% and 43% on an average, respectively with respect to water control. In cv. Nayanmani the decline in SDH activity was less significant amounting to 13% and 9% in average in the shoot and root respectively on arsenate treatment alone. Addition of phosphate along with arsenate exhibited less reduction in SDH activity in both the cultivars compared to arsenate treatment alone. In cv. IR64 shoot and root, the decline was 15% and 12%, while in cv. Nayanmani it was 6% and 1% in average respectively (**Figure 6**).

4.3.5. Fumarase Activity

An elevation in fumarase activity was observed under increasing concentrations of arsenic in both the test seedlings with cv. IR64 seedlings showing average 118% and 69% increase in the root and shoot respectively. Cv. Nayanmani experienced 57% and 49% elevation on an average in the root and shoot with respect

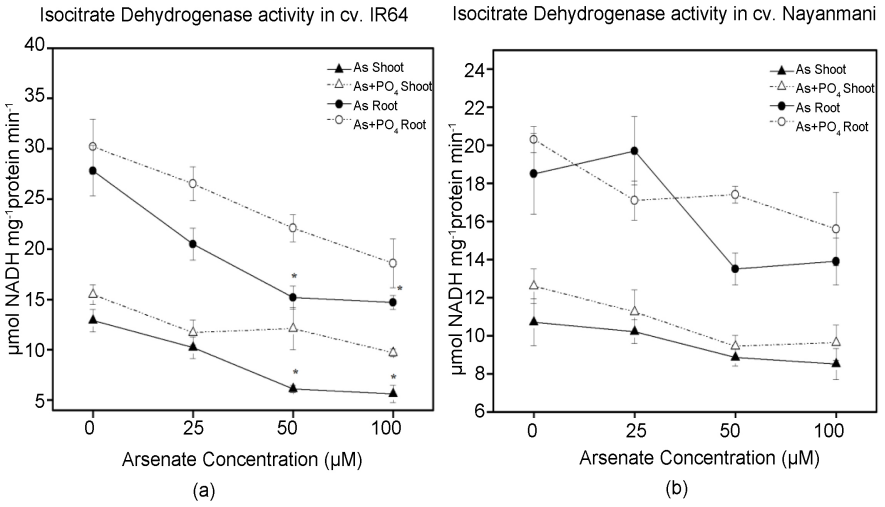


Figure 5. (a) (b) Effect of arsenate and phosphate applied singly or in combination on the Isocitrate Dehydrogenase (IDH) activity of 21 days old rice seedlings. The values are means of 3 replicas ± SE. * indicates statistically significant at $P \leq 0.05$ respectively as compared to water control.

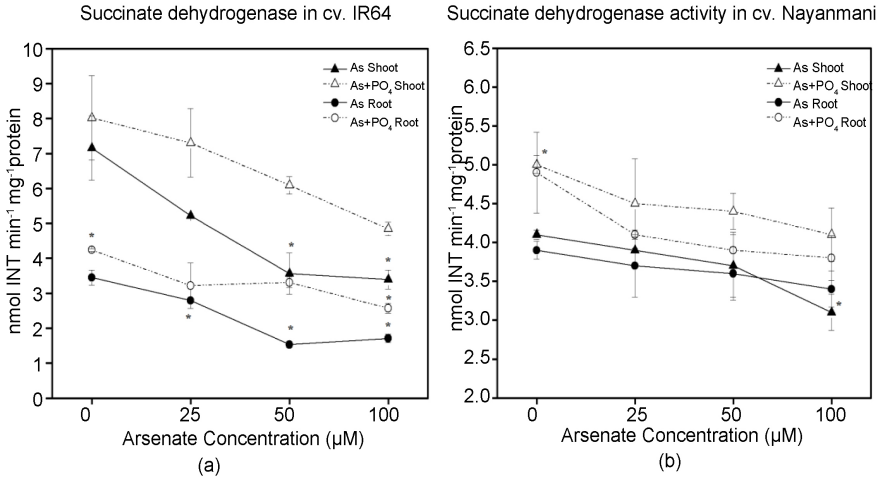


Figure 6. (a) (b) Effect of arsenate and phosphate applied singly or in combination on the succinate dehydrogenase (SDH) activity of 21 days old rice seedlings. The values are means of 3 replicas ± SE. * indicates statistically significant at $P \leq 0.05$ respectively as compared to water control.

to water control under As treatment alone. Co-application of phosphate along with arsenate resulted in less escalated fumarase activity in the test cultivars. Cv. Nayanmani showed 31% and 14% increase, while cv. IR64 experienced 44% and 18% increase on an average in the roots and shoots in comparison to water control (**Figure 7**).

4.3.6. Malate Dehydrogenase Activity

Malate dehydrogenase (MDH) activity was inhibited under As stress. Cv. IR64 experienced 37%, 52%, 67% and 21%, 28%, 42% reduction in the root and shoot respectively under increasing arsenate treatment. In cv. Nayanmani roots and

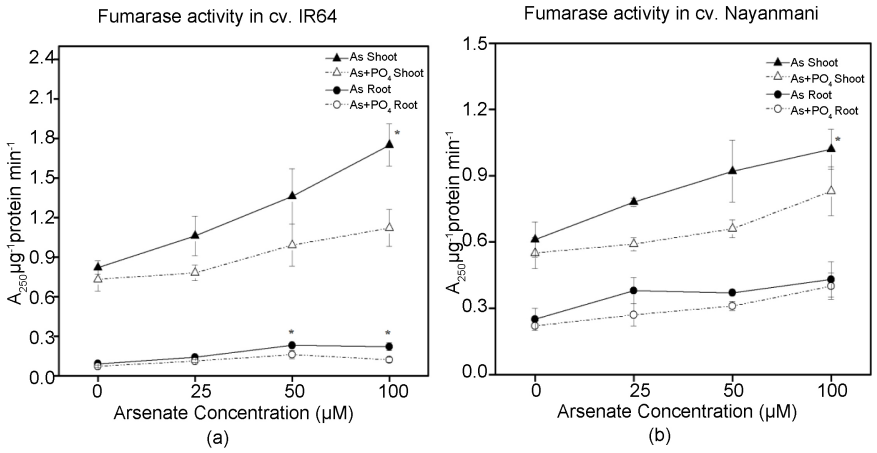


Figure 7. (a) (b) Effect of arsenate and phosphate applied singly or in combination on the Fumarase activity of 21 days old rice seedlings. The values are means of 3 replicas ± SE. * indicates statistically significant at $P \leq 0.05$ respectively as compared to water control.

shoots, this inhibition was 14%, 33%, 38% and 9%, 25%, 34% respectively in comparison to water control. Joint application of phosphate along with arsenate alleviated the toxic effect of arsenic to some extent with cv. Nayanamani root and shoot showing average 13% and 7% decrease in enzyme activity, while cv. IR64 recorded 31% and 25% inhibition in the root and shoot on an average respectively (**Figure 8**).

5. Discussion

5.1. Effect on Growth

Reduction in growth is usually observed in plants exposed to arsenic stress. The root system was much more affected than the shoots, becoming stubby, brittle and turning brown. The inhibition was stronger in roots than in shoots when exposed to arsenic because the roots are the first point of contact, as arsenate is taken up by the phosphate transporters present in the roots [28]. The metalloid also hindered root extension and proliferation. With increasing concentrations of arsenate the shoots became more stunted resulting in decreased biomass. [29] reviewed that translocation of As to the shoot drastically impedes plant growth by retarding development and biomass accumulation resulting in reduced reproductive sufficiency. Similar effect was observed in *Holcus lanatus* [30], wheat [31] and rice [32]. In our study too, the rice seedlings when exposed to increasing concentrations of arsenate, experienced much more inhibition in root length than in the shoots and this effect was more prominent in cv. IR64 than in cv. Nayanmani which showed partial tolerance to arsenic toxicity.

5.2. Effect on the Intermediates of Krebs Cycle

The formation and regulation of organic acids by TCA cycle in plants is a highly intricate phenomenon. Apart from respiration, photosynthesis and nitrogen metabolism, organic acids are said to have vital roles in helping the plant to adapt to nutrient deficiencies and metal stress [33]. We have recorded the pyru

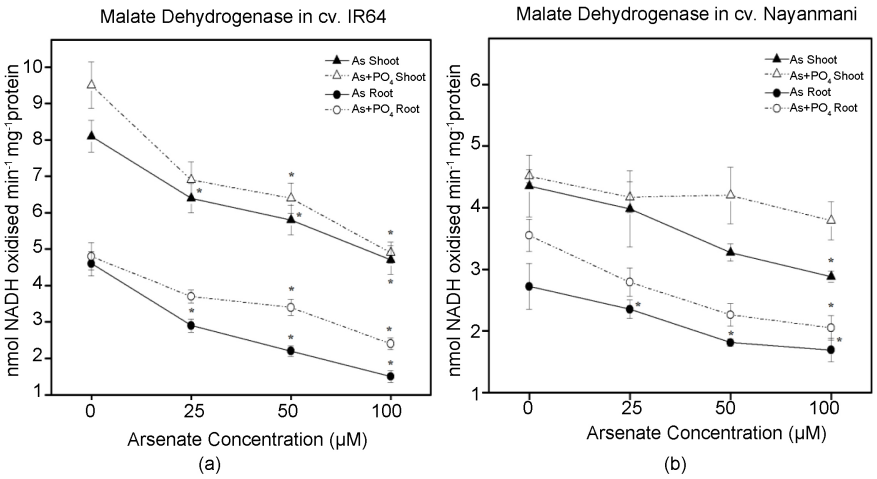


Figure 8. (a) (b) Effect of arsenate and phosphate applied singly or in combination on the malate dehydrogenase (MDH) activity of 21 days old rice seedlings. The values are means of 3 replicas ± SE. * indicates statistically significant at $P \leq 0.05$ respectively as compared to water control.

vate, citrate and malate contents of 21 days old arsenic challenged rice seedlings in our study. Pyruvate, the end product of glycolysis is decarboxylated by the enzyme pyruvate dehydrogenase (PDH) forming acetyl coenzyme A which enters the TCA cycle. Citric acid and malic acid are the intermediates in the citric acid cycle, and their synthesis and degradation are closely related with the TCA cycle [34].

In our study we observed an elevation in the organic acid contents in the test seedlings treated with sodium arsenate. The levels of pyruvate, citrate as well as malate increased considerably in cv. IR64, especially in the roots, in comparison to cv. Nayanmani which experienced relatively less elevation in its organic acid contents. Increase in citric acid and malic acid contents were also detected in two rice cultivars studied under Fe stress. Exogenous application of potassium alleviated the said stress to some extent resulting in reduced amounts of citrate and malate [35]. Similar nature of amelioration of arsenic stress has been demonstrated in our study with the joint application of phosphate along with arsenate which resulted in the production of decreased levels of organic acids.

Acids like malic, malonic, citric, fumaric and succinic among others are predominantly found in the root exudates of plants and seem to have a major role in solubilization of nutrients [36]. Organic acid accumulation has been confirmed as one of the mechanisms of stress tolerance in crop roots [37]. Thus, the presence of elevated levels of organic acids indicate the degree of metal toxicity in our test seedlings. In support of our results, [38] also recorded organic acid accumulation in mungbean seedlings under salinity stesss. It has also been documented that several wheat species excrete organic acids from their root apices due to phosphorus (P) deficiency [39]. [40] concluded that in snapbeans under aluminium stress, P deficiency caused by the formation of Al-phosphate precipitates led to the citric acid secretion. On application of phosphate along with arsenate, the increase in the organic acid contents was much less than arsenate

treatment alone indicating that phosphate was able to alleviate the metal toxicity to some extent.

5.3. Effect on the Activities of Krebs Cycle Enzymes

The mitochondrial electron transport chain is chiefly responsible for the production of intracellular ROS. As a result, the metabolic pathways in the mitochondria become sensitive to the oxidative changes in the intracellular environment. Studies suggest that mitochondria may act as "sensors" in the comprehensive stress response in plants [41]. The Krebs' cycle reactions take place in the matrix of the mitochondria and is composed of a series of enzyme catalyzed reactions. It is the key metabolic pathway that unifies carbohydrate, fat, and protein metabolism. In our study, arsenate stress caused inhibition in the activities of the dehydrogenases of the TCA cycle to variable extents in the tested cultivars, while the activities of citrate synthase and fumarase were induced.

Pyruvate dehydrogenase (PDH), occurring outside TCA cycle, is considered as a primary target for the toxic action of arsenic. Any disruption of action for this enzyme undermines the ability of the cell to meet its energy requirements and could therefore, result in cellular damage and death [42]. A reduction in the activity of PDH has been observed in the test seedlings under arsenate stress, which supports our result of increased accumulation of pyruvate in the above. The level of accumulation of pyruvate as well as the decrease in the activity of PDH was more in IR64 in comparison to cv. Nayanmani.

Citrate synthase (CS) catalyzes the combination of oxaloacetate and acetyl CoA to produce citrate while isocitrate dehydrogenase (IDH) catalyzes the oxidative decarboxylation of isocitrate in the TCA cycle. In our study, an increase in the activity of CS was observed especially in the roots of the test seedlings under arsenate stress. Whereas, inhibition in IDH activity was seen under the same conditions. In a recent study, out of the three IDH isozymes (d1 d2 and d3) isolated from cucumber roots, two of them (d2 and d3) showed diminished activity under hypoxic stress [41]. Both increase in CS activity and reduction in IDH activity supports the accumulation of citrate as found earlier in the arsenate treated seedlings. [35] also found similar results when they subjected different rice cultivars to Fe stress. Thus, adaptation of plants to metal stress is accompanied by rearrangements in oxidative metabolism as reflected by changes in metabolic pathways resulting in the accumulation of substrates that have an osmotic balancing effect and also results in the alterations of enzymes involved in oxidative metabolism. Joint application of phosphate along with arsenate altered the activities of the above mentioned enzymes in respect of arsenate treatment alone, with the effect being more pronounced in cv IR64.

Mitochondria are major producers of cellular ROS in plants under arsenic challenged conditions, resulting in the accumulation of H_2O_2 and thereby inhibiting succinate dehydrogenase (SDH) activity. With arsenate treatment, a reduction in SDH activity was observed in the test seedlings with maximum inhibition experienced in cv. IR64 and least in Nayanmani. Upon entering the cells

the toxic metals react with the metabolically active mitochondria resulting in the shrinkage of cytochrome c oxidase and succinate dehydrogenase formation. Thus, the persistant decline in succinate dehydrogenase activity in the present study along with increase of arsenate dose is consistent with this hypothesis. [43] found similar decrease in SDH activity in *Aspergillus niger* under As toxicity. [41] also reported reduced SDH activity in cucumber roots under hypoxic stress.

The activity of malate dehydrogenase (MDH) considerably decreased in both root and shoot of the arsenate treated test seedlings, with the effect being more pronounced in cv. IR64 than in cv. Nayanmani. [35] reported reduction in MDH activity in rice leaves under Fe stress. The inhibitory effect of arsenic stress on the enzyme is through conformational changes [44] but increased concentration of the substrate i.e. malate counteracted the damaging effects caused by arsenic and thus may have a protective role [45] under arsenate treatment. Malate is known to regulate the pH and limit enzymatic browning by chelating and hindering the phenol enzyme activity. On application of arsenate with phosphate the MDH activity improved in comparison to the arsenate treatment alone. Increase in the malate content under arsenic stress also supports the elevation in the activity of the fumarase enzyme in both the cultivars, but to variable extents. Joint application of phosphate along with arsenate witnessed a significant decrease in the fumarase activity, which is also correlated with the level of malic acid measured during these treatments.

6. Conclusion

Arsenic pollution of the groundwater is an impending threat in the agriculture based countries of South East Asia as well as many other parts of the world. In our study, arsenic toxicity led to considerable reduction in growth in both the test seedlings, to variable extents. The cultivar Nayanmani showed less morphological alterations in comparison to cv. IR64, which suggests a partial tolerance of the former towards arsenic stress. The intermediates as well as the enzymes of TCA cycle also showed major alterations under arsenic challenged conditions. The activities of pyruvate dehydrogenase, isocitrate dehydrogenase, succinate dehydrogenase and malate dehydrogenase were significantly inhibited, while the levels of organic acids as well as the activities of citrate synthase and fumarase enzymes were enhanced under arsenic treatment alone. These changes were however; more prominent in cv. IR64 than in cv. Nayanmani which supports our earlier morphological result that the latter is partly tolerant to the said metalloid. On joint application of phosphate along with arsenate, the harmful effects of arsenic were ameliorated in both the test seedlings, which showed better growth as well as respiration. Thus, application of external phosphate in arsenic rich soils can be suggested as a possible remedial strategy to combat arsenic contamination in rice fields.

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