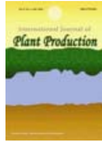

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Phosphate supply as a promoter of tolerance to arsenic in pearl millet

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Abstract

Many soils are contaminated by heavy metals, with arsenic posing serious environmental threat. Enrichment of soil with phosphate is believed to reduce the arsenic toxicity. However, only a few attempts have already been conducted towards understanding the precise role of phosphate in controlling As toxicity. Moreover, there is no adequate information on the effect of phosphate on As-induced oxidative stress, a major contributor of As toxicity in plant system. The present study aims to determine the effect of varying concentrations of phosphate on As-mediated oxidative stress in pearl millet (*Pennisetum typhoides*). Our study revealed that a concentration of 100 µM of phosphate was able to protect the test plant from As (100µM)-mediated oxidative stress, as evident from the data on growth and lipid peroxidation. This novel finding was further supported by the increased chlorophyll content of the test plant raised in phosphate supplemented media. Phosphate-mediated improvement of antioxidant enzymes, such as SOD (superoxide dismutase), CAT (catalase), and APX (peroxidase), could be a crucial factor in regulating As-induced oxidative stress in pearl millet. The results of the present study suggest that availability of phosphate in the surrounding media has a clearly pivotal role in the generation of oxidative stress in *P. typhoides* exposed to As.

Keywords: Heavy metals; *Pennisetum typhoides*; Phytoremediation.

Introduction

Arsenic (As) contamination in food chain is one of the most serious environmental problems. It is a highly toxic, non-essential element, usually

accumulates in the plant tissues through the phosphate transporters as a phosphate analogue. However, arsenic is a non-redox active element but it has been reported that arsenic produces reactive oxygen species (ROS) during electron transfer in reduction of arsenate to arsenite via oxidation of NADPH in plants. Biochemical studies in several species such as *Holcus lanatus*, red clover, oat, mung bean and rice (Hartley-Whitaker et al., 2001; Stoeva and Bineva, 2003; Singh et al., 2007) and proteome analysis of maize (Requejo and Tena, 2005) have already suggested that oxidative stress is a major contributor of arsenic toxicity in plants. Diverse responses of antioxidant defense system against As have also been reported in various plant models. It has been reported that superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase and glutathione reeducates were stimulated in crops upon exposure to arsenic (Hartley-Whitaker et al., 2001; Singh et al., 2007).

The toxicity of arsenic (like other heavy metals) depends on many factors such as pH and nutrient supply (John et al., 2009). Among all factors, phosphate level of soil or nutrient media is one of the most important that influence plant growth and arsenic uptake. Tu and Ma (2003) have reported competitive effect between arsenate and phosphate in Chinese break fern. Arsenate mimics phosphate inside the plants, due the similar electronic configuration and chemical properties, and there by replacement of phosphate from ATP, it forms unstable complex ADP-As leading to disrupted energy flow in cells (Quaghebeur and Rangel, 2003).

Arsenate is also known to repress the gene involved in the phosphate starvation responses. Catarecha et al. (2007) have been reported that arsenate may mislead the phosphate sensors and interfere with phosphate signaling mechanism in *Arabidopsis thaliana.* Phosphate is a macronutrient and necessary for photosynthesis, energy transfer, flowering and root development. Therefore, the interaction between phosphate and arsenate might be an important factor to affect arsenic-induced oxidative stress in plants. Most of the previous studies on arsenic and phosphate interaction were mainly carried out in a narrow range of arsenic uptake and transport. However, limited reports have been available about influence of phosphate on arsenic induced oxidative stress in crops.

Several studies indicate that phosphate may partially protect the membrane from arsenic induced oxidative stress in chickpea, wheat, and other species (Geng et al., 2006; Wang et al., 2007). Phosphate starvation causes decline in antioxidant enzyme activity of superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) in wheat plants exposed to

arsenic (Wang et al., 2007). However, it has to be noted that the most of the studies have been carried out in soil, where As toxicity is influenced by environmental factors such as soil type, availability of phosphate and arsenate, nutrient supply and soil pH (Tu and Ma, 2003).

The entire mechanism of phosphate mediated mitigation of arsenic induced oxidative stress is not understood. Therefore, the aim of the study was to examine aspects of the oxidative stress and antioxidant defense system in hydroponically grown pearl millet (*P. typhoides*) exposed to arsenic. The effect of phosphate supply on oxidative stress and related metabolic activities were also studied. Another novel parameter of the present approach was that only aerial plant material was used for the biochemical assay, since the generation of ROS during light reaction is an important factor in oxidative stress. Moreover, aerial parts of crops bear edible parts; therefore studying aerial part may provide practically important information.

Materials and Methods

Plant material

Seeds of pearl millet (*P. typhoides*) were collected from a crop field in Krishi Vigyan Kendra, Banasthali University (Rajasthan), located in North-eastern part of the state (75.19' to 76.16' E; 25.41' to 26.24' N). Seeds were surface sterilized with NaClO (0.1%). Thereafter, they were soaked in water for overnight at 4 ^{o}C and then transferred to Petri plates having two layers of filter paper (Whatman N_2^{o}) moistened with water. Petri plates containing seeds were incubated in the dark at room temperature until emergence of radicles.

Experimental details

The hydroponic systems used are common in biological research, in order to find most accurate results because it is possible to completely control the nutrient level and other environmental factors. In our study, it was used a static solution culture technique among the subtypes of solution culture (Static culture, continuous flow and aeroponics). Hydroponic experiments were conducted twice with the same plant material, in Banasthali University of Rajasthan during the summers of 2009 and 2010, under the same conditions. Pearl millet seedlings at 2-leaf stage were transplanted on perforated polystyrene plate floating on aerated 200 mL modified Hoagland's nutrient solution (Hoagland, 1950) in plastic pots. The

nutrient solution was renewed three times during experiment. The nutrient solution used for both experiments comprised of 1.0 mM KNO_3, 0.4 mM NH_4NO_3, 0.8 mM $Ca(NO_3)$, 0.4 mM $MgSO_4.7H_2O$, 2 µM $MnCl_2.4H_2O$, 9 µM H_3BO_3, 0.2 µM $ZnSO_4.7H_2O$, 0.06 µM $CuSO_4.5H_2O$, 0.02 µM H_2MoO_4. H_2O and 9 µM EDTA-Fe. The As was added as $Na_2HAsO_4.7H_2O$. The nutrient solution pH (6.8) was adjusted with 1N HCl and 1N NaOH. All chemicals were of analytical grade. After one day acclimatization, roots of the test plants were treated with stock solution of As prepared from Na_2HAsO_4 to obtain 10, 25, 50, 100 and 200 μM As in experimental pots. The pots without any As enrichment were considered as control. The experiment was conducted in a Complete Randomized Design (CRD) and treatments were replicated 3 times. The triplicates of each treatment were placed in a growth chamber at 27±2 ºC under a 12 h daylight period (120 μmol m^{-2} s^{-1}) during 10 days. Light was provided by 4 high pressure sodium lamps (Vialox NAV-T 400 4Y, Osram, Gmbh, Munich, Germany). After 10 days, the plants were harvested and dose-response experiment over 10 d was conducted to examine oxidative damage in test plants. Shoot and root length was measured to study the effect of As on plant growth, after 10 days exposure to As. Highest root length is expected in the beginning and at the end of the exposure period, since root elongation is one of the most sensitive indicators of metal toxicity.

Quantitative analysis of arsenic uptake

Arsenic uptake by test plant was measured by determining the Intra cellular arsenic content. For this, test plants were harvested at 10th day of arsenic exposure. After washing in 10 mL of 2 mM EDTA for 10 min to remove the metal ions adhering to the root surface, plant material was rinsed in deionized water and oven dried. Separated root and shoot of test plants were ground into fine powder and 0.5 gm test material was digested with nitric acid on a hot plate at 80 ^{o}C until the solution became colorless. The residue was dissolved in distilled water and the final volume adjusted to 10 mL. The digested samples were analyzed for content with an atomic absorption spectrophotometer (Shimadzu AA-6300, Japan).

Lipid peroxidation

Lipid peroxidation was estimated by measuring the formation of malondialdehyde (MDA) with 2-thiobarbituric acid (De Vos et al., 1989).

The aerial part of *P. typhoides* seedlings were extracted in 5 mL of 0.25% (w/v) TBA in 10% (w/v) trichloroacetic acid and heated at 95 ºC for 30 min. After cooling in ice, the mixture was centrifuged at 5000 rpm for 10 min. MDA was determined by subtracting absorbance of supernatant at 600 nm from that at 532 nm and using an absorbance coefficient of 155 mM^{-1} cm^{-1} (Kwon et al., 1965).

H_2O_2 determination

Test plants were homogenized with 5.0 ml of TCA (0.1%, w/v) in an ice bath, and the homogenate were centrifuged at 12.000 g for 15 min. To 0.5 mL of supernatant, 0.5 mL phosphate buffer (pH 7.0) and 1.0 mL of potassium iodide (1 M) were added. The absorbance of the mixture was measured at 390 nm. The extinction coefficient of H_2O_2 was 0.28 $\mu M^{-1}cm^{-1}$.

Photosynthetic pigments

The photosynthetic pigment from As treated and control pearl millet seedlings were extracted in 80% acetone (5 mL) and homogenate was centrifuged at 5000 rpm for 10 min. The concentrations of chlorophylls (Chl) a and b and total chlorophyll content were determined spectrophotometrically using two wavelengths: 663 and 645 nm, respectively (Arnon, 1949).

Enzyme assay

The aerial parts of treated and control seedlings were homogenized in pre-chilled mortar and pestle. Extraction mixture contained 1 mM EDTA, 0.05% Triton X-100, 2% (w/v) Polyvinylpyrrolidone and 1 mM ascorbate in 50 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 14000 rpm for 20 min at 4 ºC, the resulting supernatant was kept at -20 ºC and used for the measurement of SOD, APX and CAT activities. The protein concentration of homogenates was determined according to the method described by Lowry et al. (1951), using bovine serum albumin as a standard.

Superoxide dismutase (SOD) EC 1.15.1.1

SOD activity of As treated and control *P. typhoides* was determined by measuring the inhibition of photochemical reduction of nitroblue tetazolium

(NBT) at 560 nm as suggested by Beauchamp and Fridovich (1971). 0.1 mL of enzyme extract was added to 2.5 mL of reaction mixture contained 0.1 mM EDTA, 13 mM methionine and 75 mM NBT in 50 mM phosphate buffer (pH 7.8), after that 0.4 mL riboflavin (13 μM) was added in the dark. Thereafter, the mixture was placed under fluorescent light for 30 min. A blank (without enzyme extract) was also prepared. The photoreduction of NBT (formation of blue formazan) was measured at 560 nm. The amount of reduced NBT was calculated using the absorbance coefficient 100 mM^{-1} cm^{-1}.

Ascorbate peroxidase (APX) EC 1.11.1.11

The activity of APX was measured by estimating the rate of ascorbate oxidation at 290 nm. 0.1 mL of enzyme extract was added to 2.8 mL of reaction mixture containing 0.5 mM ascorbic acid, 0.1 mM EDTA in 50 mM phosphate buffer (pH 7.0) and absorbance was taken after 15 sec at 290 nm. Absorbance was again recorded after adding 0.1 mL H_2O_2 (0.1 mM). The enzyme activity was calculated using extinction coefficient 2.8 $mM^{-1}cm^{-1}$.

Catalase (CAT) EC 1.11.1.6

CAT activity of the test plants was assayed by the method as given by Clairbone (1985). 0.1 mL of enzyme extract was added in 2.8 mL of 50 mM phosphate buffer (pH 7.0) and absorbance was taken at 240 nm. Subsequently, 0.1 mL of 3.125 mM H_2O_2 was added and absorbance was recorded after every 30-90 sec. The extinction coefficient for CAT was 0.039 mM cm^{-1} (Aebi, 1984).

Relationship between phosphate supply and arsenic induced oxidative damage

Experiments were conducted to explore the effect phosphate level during arsenic induced oxidative stress. For this purpose the test plants were treated with 0, 25, 50, 100 and 250 μM phosphates and 100 µM arsenic under the conditions describe earlier. Previous experiment showed approximately 50% inhibition of growth of the test plant at 100 μM arsenic (data not shown). The role of phosphate in mitigating arsenic–induced oxidative stress was evaluated by measuring growth, lipid peroxidation, H_2O_2 content, antioxidant enzyme activities of SOD, CAT and APX according to the procedure described above.

Statistical analyses

All the described experiments were set up in a completely randomized design with three replications (pots), while each experiment was conducted twice. Data were processed using STATISTICA 7.0 software (StatSoft Inc., Tulsa, USA). ANOVA with post hoc Duncan's multiple range test (P=0.05) was applied to compare the means of each parameter for all the treatments. Because the ANOVAs indicated no significant experiment by treatment interaction, data were averaged across experiments, while all data were tested for normality and variance before further analyses.

Results

The effect of arsenic on plant growth is shown in Table 1. The root length of *P. typhoides* inhibited by 15%, 26%, 34%, 36% and 63% at 10, 25, 50, 100 and 200 μM As. However, the inhibitory effect of arsenic on shoot length was significantly lower, since the shoot length of test plant was reduced by 0.4%, 11%, 19%, 22% and 40% at 10, 25, 50, 100 and 200 μM As concentration, respectively. Only highest tested concentrations of arsenic (100 and 200 μM) were able to induced oxidative stress in *P. typhoides.* Amount of MDA content was 2 times higher in plants exposed to 100 and 200 μM As than that of control. Almost similar pattern was followed by another oxidative stress indicator i.e. H_2O_2. Exposure of test plant to arsenic causes concentration dependent enhancement of thiole content in test plants. Our results further show that the level of thiole remained unchanged up to 50 μM of arsenic treatment and thereafter it was significantly ($P \leq 0.05$) increased in pearl millet.

Concentration dependent decline was observed in chlorophyll content of test plant exposed to arsenic. Chlorophyll *a* of test plant decreased by 24%, 22%, 29%, 31% and 55% and total chlorophyll by 30%, 32%, 34%, 39% and 62% at 10, 25, 50, 100 and 200 μM arsenic, respectively (Table 2). Concentration dependent decline was also observed for Chlorophyll *b* in test plants exposed to increasing concentrations of arsenic. Test plant showed 38%, 34%, 41%, 44% and 65% reduction in carotenoid content at 10, 25, 50, 100 and 200 μM arsenic concentrations, respectively.

Table 1. Effect of arsenic (As) treatments on *P. typhoides* growth (root and shoot length), As accumulation, lipid peroxidation, H_2O_2 and thiol content. Values represent the means of the two experiments, followed by the standard errors. For each parameter, means followed by the same low case letters are not significantly different according to Duncan's multiple range test at P≤0.05.

As treatment (μM)	As content (μM gm^{-1})	Root length (cm)	Shoot length (cm)	MDA (nM μg^{-1})	H_2O_2 (μM μg^{-1})
0	0.00 ± 0.00^{c}	8.1 ± 0.02^{a}	10.9 ± 0.03^{a}	1.433 ± 0.05^{c}	0.020 ± 0.00^{b}
10	0.52 ± 0.02^{b}	6.9 ± 0.01^{ab}	10.9 ± 0.02^{a}	2.137 ± 0.01^{bc}	0.021 ± 0.01^{b}
25	0.66 ± 0.04^{b}	6.0 ± 0.01^{ab}	9.7 ± 0.01^{ab}	2.451 ± 0.03^{bc}	0.028 ± 0.01^{ab}
50	0.89 ± 0.03^{ab}	5.4 ± 0.03^{ab}	8.8 ± 0.01^{ab}	2.896 ± 0.04^{ab}	0.037 ± 0.01^{a}
100	0.93 ± 0.01^{ab}	5.3 ± 0.01^{ab}	8.6 ± 0.01^{ab}	3.568 ± 0.02^{ab}	0.040 ± 0.02^{a}
200	1.34 ± 0.01^{a}	3.1 ± 0.01^{b}	6.6 ± 0.01^{b}	4.794 ± 0.01^{a}	0.042 ± 0.02^{a}

Table 2. Changes in the amount of photosynthetic pigments of *P. typhoides* exposed to various concentrations of arsenic (As) for 10 days. Values represent the means of the two experiments, followed by the standard errors. For each parameter, means followed by the same low case letters are not significantly different according to Duncan's multiple range test at P≤0.05.

As treatment (µM)	Chlorophyll *a* (mg μg^{-1})	Chlorophyll *b* (mg μg^{-1})	Total chlorophyll (mg μg^{-1})	Carotenoid (mg μg^{-1})
0	0.0104 ± 0.001^{a}	0.0048 ± 0.001^{a}	0.0153 ± 0.003^{a}	0.1440 ± 0.027^{a}
10	0.0079 ± 0.000^{a}	0.0024 ± 0.001^{b}	0.0107 ± 0.001^{a}	0.0906 ± 0.006^{b}
25	0.0081 ± 0.001^{a}	0.0025 ± 0.001^{b}	0.0103 ± 0.001^{a}	0.0952 ± 0.016^{b}
50	0.0073 ± 0.001^{b}	0.0027 ± 0.001^{b}	0.0101 ± 0.001^{ab}	0.0852 ± 0.009^{b}
100	0.0072 ± 0.001^{b}	0.0021 ± 0.001^{b}	0.0093 ± 0.001^{b}	0.0811 ± 0.014^{b}
200	0.0046 ± 0.000^{c}	0.0012 ± 0.001^{c}	0.0059 ± 0.001^{c}	0.0501 ± 0.003^{c}

The SOD activity of *P. typhoides* was increased with increasing concentration of arsenic. The SOD activity of test plant enhanced by 2 folds after test plants exposed to 10, 25, 50 and 100 µM arsenic treatment. Above these concentrations (i.e. 200 µM), arsenic treatment resulted to a lower level of SOD activity in test plants (Table 3). The induction of CAT activity ranged from 75-100% up to 100 μM level of arsenic, thereafter increased concentration of arsenic caused lower level of CAT activity in *P. typhoides* (Table 3).

Antioxidant enzyme activity of APX increased by 18%, 25% and 160% in plants exposed to 10, 25 and 50 μM arsenic, respectively. Moreover, APX activity was dramatically affected in test plants exposed to 100 and 200 μM arsenic treatment.

Table 3. Changes in the antioxidative enzymes activities of *P. typhoides* exposed to increasing concentrations of arsenic (As) for 10 days. Values represent the means of the two experiments, followed by the standard errors. For each parameter, means followed by the same low case letters are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

As treatmnent (µM)	SOD (U μg^{-1})	CAT (U μg^{-1})	APX (U μg^{-1})
0	0.048 ± 0.017^{b}	17.646 ± 1.89^{c}	0.325 ± 0.016^{ab}
10	0.155 ± 0.001^{a}	31.126 ± 2.04^{a}	0.383 ± 0.015^{ab}
25	0.165 ± 0.000^{a}	32.786 ± 2.35^{a}	0.408 ± 0.044^{ab}
50	0.171 ± 0.000^{a}	37.355 ± 1.46^{a}	0.842 ± 0.029^{a}
100	0.154 ± 0.000^{a}	24.870 ± 2.16^{b}	0.403 ± 0.025^{ab}
200	0.129 ± 0.009^{a}	28.822 ± 0.71^{ab}	0.281 ± 0.023^{b}

The effects of phosphate supply on arsenic uptake and plant growth are shown in Table 4. Increasing concentration of phosphate causes decline in root growth inhibition. Arsenic concentration in test plants significantly increased by elevated level of phosphate. The arsenic transport ratio increased from 0.004 to 1.959, depending on the elevated level of P (Table 4).

Table 4. Effect of various concentrations of phosphate on growth and arsenic (As) content in aerial parts of *P. typhoides* exposed to arsenic (100 μM). Values represent the means of the two experiments, followed by the standard errors. For each parameter, means followed by the same low case letters are not significantly different according to Duncan's multiple range test at $P \leq 0.05$.

Phosphate supply (μM)	As treatment (μM)	Root length (cm)	Shoot length (cm)	Root As ($\mu M\ g^{-1}$)	Shoot As ($\mu M\ g^{-1}$)
0	0	5.71 ± 0.30^{cd}	5.04 ± 0.58^{c}	0.000 ± 0.00^{d}	0.000 ± 0.00^{d}
	100	5.47 ± 0.65^{d}	4.77 ± 1.24^{c}	0.545 ± 0.00^{a}	0.517 ± 0.00^{a}
25	0	6.18 ± 0.82^{c}	6.60 ± 1.16^{b}	0.000 ± 0.00^{d}	0.000 ± 0.00^{d}
	100	5.90 ± 1.50^{c}	5.70 ± 0.96^{bc}	0.344 ± 0.00^{b}	0.296 ± 0.00^{b}
50	0	8.72 ± 0.57^{a}	7.85 ± 1.60^{a}	0.000 ± 0.00^{d}	0.000 ± 0.00^{d}
	100	7.12 ± 1.30^{ab}	5.71 ± 0.71	0.223 ± 0.00^{bc}	0.214 ± 0.00^{bc}
100	0	8.91 ± 1.60^{a}	8.07 ± 1.47^{a}	0.000 ± 0.00^{d}	0.000 ± 0.00^{d}
	100	7.74 ± 1.14^{ab}	5.97 ± 1.03^{bc}	0.142 ± 0.01^{c}	0.160 ± 0.00^{c}
250	0	9.90 ± 1.10^{a}	8.40 ± 1.71^{a}	0.000 ± 0.00^{d}	0.000 ± 0.00^{d}
	100	9.10 ± 1.44^{a}	6.44 ± 1.38^{b}	0.094 ± 0.00^{c}	0.098 ± 0.00^{c}

Table 5 shows that arsenic treatment causes decrease in the amount of photosynthetic pigments, while it was observed that plants treated with 100 and 250 μM phosphate and 100 μM arsenic had higher level of photosynthetic pigments than plants grown on substrate with a lower level of phosphate. It was further observed that there was a significant decrease in MDA content in pearl millet plants treated with 100 μM phosphate. Similarly, H_2O_2 content was also significantly decreased in plants exposed to 100 μM arsenic with100 μM of phosphate.

Table 5. Effect of various concentrations of phosphate on photosynthetic pigments, lipid peroxidation, and H_2O_2 content in aerial parts of *P. typhoides* exposed to arsenic (100 µM). Values represent the means of the two experiments, followed by the standard errors. For each parameter, means followed by the same low case letters are not significantly different according to Duncan's multiple range test at P=0.05.

Phosphate supply (µM)	As treatment (µM)	Chl a (mg µg^{-1})	Chl b (mg µg^{-1})	Total Chl (mg µg^{-1})	Carotenoid (mg µg^{-1})	MDA (nM µg^{-1})	H_2O_2 (µM µg^{-1})
0	0	0.021 ± 0.002^{b}	0.045 ± 0.002^{b}	0.120 ± 0.001^{a}	0.121 ± 0.001^{bc}	1.65 ± 0.53^{b}	0.013 ± 0.001^{ab}
	100	0.007 ± 0.001^{b}	0.002 ± 0.001^{d}	0.009 ± 0.002^{d}	0.109 ± 0.002^{c}	2.85 ± 0.08^{a}	0.028 ± 0.001^{a}
25	0	0.113 ± 0.001^{a}	0.069 ± 0.002^{ab}	0.105 ± 0.001^{ab}	0.144 ± 0.001^{b}	1.98 ± 0.45^{ab}	0.014 ± 0.002^{ab}
	100	0.015 ± 0.001^{b}	0.003 ± 0.000^{d}	0.017 ± 0.001^{cd}	0.134 ± 0.001^{bc}	3.21 ± 0.47^{a}	0.029 ± 0.002^{a}
50	0	0.115 ± 0.002^{a}	0.079 ± 0.001^{a}	0.117 ± 0.001^{a}	0.148 ± 0.000^{b}	2.93 ± 0.58^{a}	0.010 ± 0.001^{ab}
	100	0.033 ± 0.000^{b}	0.003 ± 0.003^{d}	0.035 ± 0.000^{c}	0.139 ± 0.013^{b}	3.53 ± 0.08^{a}	0.020 ± 0.003^{a}
100	0	0.116 ± 0.001^{a}	0.086 ± 0.001^{a}	0.133 ± 0.001^{a}	0.177 ± 0.012^{a}	1.26 ± 0.29^{bc}	0.007 ± 0.001^{b}
	100	0.065 ± 0.002^{ab}	0.023 ± 0.001^{c}	0.078 ± 0.002^{b}	0.173 ± 0.002^{a}	0.90 ± 0.75^{c}	0.012 ± 0.002^{ab}
250	0	0.160 ± 0.001^{a}	0.100 ± 0.002^{a}	0.157 ± 0.001^{a}	0.150 ± 0.001^{b}	0.87 ± 0.36^{c}	0.007 ± 0.001^{b}
	100	0.114 ± 0.001^{a}	0.024 ± 0.003^{c}	0.115 ± 0.002^{a}	0.147 ± 0.010^{b}	1.20 ± 0.72^{bc}	0.011 ± 0.002^{ab}

Phosphate treatment had a clear effect on antioxidant enzyme activity of SOD, since treatment with 100 and 250 μM phosphate resulted to the highest levels of SOD activity (up to 7 times higher than the control plants). In contrast to SOD activity, a different pattern was exhibited by CAT activity. Indeed, a rather gradual increase in CAT activity was noticed in test plats supplied up to 100 μM phosphate. The CAT activity declined in plants treated with 250 μM phosphate, while there was a similar trends in APX activity, since 250 μM phosphate resulted to a lower APX activity than 100 μM phosphate.

Discussion

In the present study the arsenic-induced oxidative stress was evaluated in *P. typhoides*, while the potential effects of varying concentration of phosphate on As-mediated oxidative stress were also evaluated. Our results are in accordance with previous studies on ferns and chickpea, supporting the suppressive role of phosphate to arsenic uptake (Tu and Ma, 2003, Gunes et al., 2009). Until now, the effects of phosphate on arsenic toxicity in a hydroponic system and in higher plants have not adequately studied. Plant growth is a cumulative appearance of disorders in a cellular environment. Arsenic treatment adversely affects the plant growth that we have measured in terms of root and shoot length. Roots are the first points of contact with toxic element and accumulate more metal than shoots. Furthermore, arsenic and phosphate share similar ionic structure, and thus As is considered as analogous to phosphate, which is an essential macronutrient for plants. Our results suggest that increased supply of phosphate provided protection to the plant system and that effect appears in the form of a reduced amount of growth inhibition during arsenic toxicity.

Arsenic toxicity leads to decrease of the amount of photosynthetic pigments in *Pennisetum typhoides*. It has already been reported that arsenic causes a reduction of chlorophyll biosynthesis and damage to chloroplast membrane which may further lead to a decline in chlorophyll and carotenoid content of plants (Jain et al., 1997). Our results highlight the crucial role of phosphate supply, since it gradually reduced the arsenic toxicity and improved the level of photosynthetic pigments.

Our data have also shown that arsenic induces oxidative stress as evident from concentration dependant increase in oxidative stress indicators i.e. MDA and H_2O_2 content of the test plant. This effect of As is consistent with

previous reports in other arsenic treated crops, such as red clover, bean, mung bean and rice (Hartley-Whitaker et al., 2001; Singh et al., 2007). The study of the response of antioxidative enzymes is considered as a common approach for several other heavy metals, too (Benzarti et al., 2010). Previous studies with soil culture experiment suggest that the level of lipid peroxidation had a significant decline with higher phosphate supply during arsenic mediated oxidative stress (Gunes et al., 2009). Similarly, a remarkable decline in MDA and H_2O_2 content was observed in *P. typhoides* exposed to 100 μM phosphate concentrations. The response of antioxidant defense enzyme during arsenic toxicity and gradual supply of phosphate revels confirm that phosphate has a prominent role to upgrade antioxidant defense system during arsenic stress. The increased SOD activity in response to arsenic toxicity which was also observed in the present study is in agreement with reports in hyperaccumulator fern species, maize and tolerant clone of *Holcus lanatus* (Hartley-Whitaker et al., 2001; Srivastava et al., 2006). Moreover, supply of phosphate significantly increased the stimulation of SOD activity and it might be expected that P enables the system to encounter with arsenic toxicity. Further application of P enhances the CAT activity during arsenic mediated oxidative stress. Although application of P at a high rate limits the stimulation of catalase activity in most cases, a significant effect of 100 μM phosphate supply on CAT stimulation was observed in *P. typhoides* exposed to arsenic. Our data show an improvement of catalase activity in plants raised in phosphate enriched medium. Catalase is an important antioxidant enzyme responsible for the detoxification of H_2O_2 during oxidative stress, while earlier reports have also suggested that catalase in maize and wheat was increased when plants were exposed to arsenate (Wang et al., 2007).

Arsenic induces APX enzyme activity as observed in the present study. Further Phosphate supply alters the antioxidant enzyme activities of APX enzyme as activity increases up to 100 µM phosphate level whereas higher tested concentrations of phosphate cause declined in APX activity. Regarding the interaction of arsenic with phosphate, it has to be noted that 100 μM phosphate can practically protect the plant against arsenic toxicity. This finding is in full accordance with previous studies in other crops, also supporting that increased phosphate supply leads to decline in lipid peroxidation and alteration of antioxidant enzyme activities in wheat and chickpea (Wang et al., 2007; Gunes et al., 2009).

Conclusively, the present study suggests that an efficient antioxidant defense system makes *P. typhoides* tolerant against arsenic, such as several choice crops and weeds (Srivastava et al., 2006; Gisbert et al., 2008; Mahmud at al., 2008). It has also to be taken into account that *P. typhoides* has a definitely high tolerance without any further breeding attempt, except a common agronomical practice of P supply, while several transformations are lately proposed in order to genetically engineer crops for enhanced arsenic tolerance (Maestri and Marmiroli, 2011), Therefore, pearl millet should be certainly included in a sustainable phytomanagement strategy for excessive arsenic control, such as those recently developed for several other inorganics (Chen et al., 2010). Breeding techniques and artificial selection is anticipated to further improve some of the more promising and interesting species, such as *P. typhoides*, in order to reach their desirable commercial existence (Navabpour et al., 2007). Furthermore, Phosphate mediated improvement of antioxidant enzyme activity of catalase specifies that status of phosphate in the nutrient media plays an important role in mitigation of As-mediated oxidative stress in *P. typhoides* and therefore should be further exploited.

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