

Arsenic (As) Inhibits Radicle Emergence and Elongation in *Phaseolus aureus* by Altering Starch-Metabolizing Enzymes Vis-à-Vis Disruption of Oxidative Metabolism

Shalinder Kaur · Harminder Pal Singh ·
Daizy Rani Batish · Asha Negi · Priyanka Mahajan ·
Surabhi Rana · Ravinder Kumar Kohli

Received: 12 September 2011 / Accepted: 3 November 2011 / Published online: 29 November 2011
© Springer Science+Business Media, LLC 2011

Abstract The present study investigated the effect of Arsenic (As; 5, 10, 50 μM) on protein and sugar metabolism vis-à-vis oxidative damage during early germination process and radicle emergence (at 12, 24 and 48 h stage) in *Phaseolus aureus*. As-exposure (50 μM) significantly enhanced protein content (by 40–60%), whereas carbohydrate content declined (by 31–44%) over that in the control. It was associated with a decline in the activities of proteases (47–53%), and increase in the activities of α - and β -amylases, starch phosphorylases, and acid invertases by 3.0, 2.6, 4.8, and 1.7 times after 48 h exposure to 50 μM As. The alteration in protein and carbohydrate metabolic machinery was also accompanied by As-induced reactive oxygen species (ROS)-mediated oxidative damage. As treatment enhanced malondialdehyde and hydrogen peroxide content by 46–252% and 23–216%, and hydroxyl and superoxide ion generation by 15–104% and 17–278%, respectively. As-induced lipid peroxidation and membrane disruption was confirmed by enhanced electrolyte leakage (by 49%) and reduced cell viability (by 43%). Furthermore, in response to 50 μM As, the activities of superoxide dismutases, catalases, ascorbate peroxidases, guaiacol peroxidases, and glutathione reductases increased by 77%, 70%, 116%, 43% and 120%, respectively, in radicles at 48 h stage over that in the control. The study concludes that As inhibits radicle emergence and elongation in germinat-

ing *P. aureus* seeds by altering biochemical processes related to sugar metabolism and inducing an ROS-mediated oxidative damage.

Keywords Acid invertases · As toxicity · Emerging radicles · Mung bean · ROS generation · ROS metabolism · Starch phosphorylase · Sugar metabolism

Introduction

Arsenic (As) is a serious environmental toxicant released into the environment from both natural and anthropogenic sources and causes toxicity to plants, animals and humans [1, 2]. Additionally, the extensive use of underground water for irrigation has further elevated As concentrations in the soil and plants including grains [3]. The contamination of the ground water with As has resulted in As accumulation, especially under anaerobic conditions, in Asian countries such as Bangladesh, India and China [4]. As accumulation in soil is highly toxic to plants and results in reduction in plant growth and crop yield [1]. High concentration of As competes with phosphate as a substrate and interferes with the uptake of phosphate and other plant nutrients, thereby impairing plant metabolism [5]. However, plants do not accumulate As at hazardous concentrations, rather they show symptoms of phytotoxicity before the concentrations toxic to organisms [6]. Earlier studies have shown that excess As inhibits seed germination, adversely affects growth, development and yield of plants, alters physiological processes, and induces cellular damage [7–9]. As toxicity depends on its concentration and exposure period, and the physiological state of the plant. Furthermore, the plants show a great deal of variation in response to As toxicity/As contamination.

S. Kaur · H. P. Singh (✉) · A. Negi · S. Rana
Department of Environment Studies, Panjab University,
Chandigarh 160014, India
e-mail: hpsingh_01@yahoo.com

D. R. Batish · P. Mahajan · R. K. Kohli
Department of Botany, Panjab University,
Chandigarh 160 014, India

Some fern species have been found to accumulate As in their fronds and grow well in As-contaminated soils [10]. Most of the studies investigating As toxicity have focused upon the uptake, accumulation, and the oxidative damage [7–11]. In our previous study, we reported that As adversely affected growth, induced anatomical changes and altered oxidative metabolism in roots of 7-day-old seedlings of *Phaseolus aureus* [9]. However, the mechanism underlying the adverse effects of As on plants during germination and radicle emergence remains unknown. Ma et al. [12] opined that antioxidant enzymatic machinery is upregulated within early hours of exposure to heavy metals, so it is pertinent to measure changes in emerging radicles/germinating seeds. Moreover, not much is known about the effect of As, a metalloid, on protein and sugar metabolism during early germination process and radicle emergence.

With this background, the present study investigated the impact of As on the changes in protein and sugar metabolism vis-à-vis the disruption of oxidative metabolism during early 12–48 h of germination process in mung bean. We selected mung bean as a bioassay species since it is a non-accumulator [9], and is regarded as an important bioindicator species for As contamination [13]. We investigated the effect of As (5, 10, 50 μM) on protein and carbohydrate content, activities of protein- and starch-hydrolyzing enzymes (proteases, α -amylases, β -amylases, starch phosphorylases, and acid invertases), generation of reactive oxygen species (ROS) and enzymatic antioxidants in radicles of germinating mung bean seeds at 12, 24, and 48 h after As exposure. The selected As concentrations were environmentally relevant and comparable to those present under natural conditions [14]. The oxidative damage was assessed in terms of malondialdehyde and hydrogen peroxide content, hydroxyl and superoxide ion generation, electrolyte leakage, and cell viability, whereas ROS metabolism was determined in terms of the activities of antioxidant enzymes, i.e., superoxide dismutases (SOD), catalases (CAT), ascorbate peroxidases (APX), guaiacol peroxidases (GPX) and glutathione reductases (GR).

Material and Methods

Materials

Seeds of mung bean (*P. aureus* Roxb. = *Vigna radiata* (L.) Wilczek; cv. ML-5) were procured locally from a seed store. Before use, these were disinfected using sodium hypochlorite (0.1%, w/v) for 15 min and washed under running tap water followed by distilled water thrice. As was supplied in the form of sodium arsenate (MW=312.01; purity=98.5%; procured from Loba-Chemie, Mumbai,

India). All other chemicals and reagents were of reagent grade and procured from the best available sources including Sigma-Aldrich (USA), Fluka (Switzerland), Acros (Belgium), Hi-Media (India), and Sisco Research Laboratory (India).

Treatments and Experimental Setup

Pre-imbibed (for 6 h at 25°C) seeds were equidistantly placed in Petri dishes ($\phi=15$ cm) lined with Whatman #1 filter circle moistened with 7 ml of 5.0, 10.0 or 50.0 μM of As or distilled water (as control). The selected concentrations were environmentally relevant and based upon an earlier study of Singh et al. [9]. For each treatment, ten independent Petri dish replicates with ten seeds for each treatment were maintained. The Petri dishes were kept in a completely randomized design (CRD) in a growth chamber under 16 h photoperiod of $\sim 240 \mu\text{mol m}^{-2} \text{s}^{-1}$ PFD, at 25/18 (± 2)°C, and 75 \pm 2% relative humidity. After 12, 24, and 48 h, radicles emerging from germinating mung bean seeds were cut with fresh blade and used for further biochemical studies and oxidative damage assessment.

Total Protein and Carbohydrate Content

Root tissue (200 mg) was homogenized in pre-chilled pestle and mortar with 10 ml distilled water, centrifuged at 15,000 \times g for 15 min, and the supernatant collected for the estimations. Protein content was measured spectrophotometrically at 700 nm using Folin–Ciocalteu reagent against a standard of bovine serum albumin [15]. Total carbohydrate content was estimated using anthrone reagent at 620 nm against a calibration standard of glucose [16].

Assay of Protein- and Starch-Hydrolyzing Enzyme

To extract proteases and α - and β -amylases, root tissue (200 mg) was homogenized in 10 ml of 0.1 M PO_4^{3-} buffer (pH 7). Acid invertases were extracted in 10 mM tris-maleate buffer (pH 7), whereas acid phosphatases were extracted in ice-cold 50 mM tris-maleate buffer (pH 7) containing 13 mM mercaptoethanol (at 4°C). The homogenates were centrifuged at 15,000 \times g for 30 min at 4°C in a cold centrifuge (Sigma, USA). The supernatants were collected and stored at 4°C for further enzymatic assays. An aliquot (0.5 ml) of the supernatants was used for protein estimation [15].

Proteases were assayed using casein (1% in 0.1 M phosphate buffer, pH=6) as a substrate and expressed as micrograms per hour per milligram protein. The activities of α - and β -amylases were assessed using starch as a substrate and expressed as micrograms per minute per

milligram protein [16]. Activity of starch phosphorylases was measured at 660 nm and expressed as micromol Pi per second per milligram protein [17]. Acid invertases were assayed in terms of reducing sugars produced at 620 nm against a calibration standard of glucose [18] and expressed as microgram per minute per milligram protein.

Assessment of Oxidative Damage

As-induced ROS generation was determined in terms of various stress markers such as lipid peroxidation (as MDA content), hydrogen peroxide (H_2O_2), superoxide ions ($O_2^{\cdot-}$), hydroxyl ions (OH^{\cdot}) content, cell viability, and membrane damage (as relative electrolyte leakage [REL]).

Lipid Peroxidation

Content of MDA, a major thiobarbituric acid (TBA) reactive species, was determined as per Heath and Packer [19]. Roots (100 mg) were homogenized in 5 ml trichloroacetic acid (TCA; 0.1%, w/v) in a pre-chilled pestle and mortar and centrifuged at $10,000\times g$ for 10 min. One milliliter of the supernatant was added to 4 ml TBA (0.5%, w/v, in 20% TCA). The mixture was heated at $95^\circ C$ for 30 min, cooled over ice, and then centrifuged at $10,000\times g$ for 10 min. The absorbance of the supernatant was read at 532 nm and corrected for non-specific absorbance at 600 nm. MDA content was determined by using the extinction coefficient (ϵ) of $155\text{ mM}^{-1}\text{ cm}^{-1}$ and expressed as nmol g^{-1} [11].

H_2O_2 Content

Roots (100 mg) were homogenized with 5 ml of 0.1% TCA (w/v) in a pre-chilled pestle and mortar and centrifuged at $12,000\times g$ for 15 min [11]. To 0.5 ml of supernatant, 0.5 ml of PO_4^{3-} buffer (pH 7), and 1 ml of 1 M potassium iodide were added. The absorbance of reaction mixture was measured at 390 nm. H_2O_2 content was determined by using an extinction coefficient ($\epsilon=0.28\ \mu\text{M}^{-1}\text{ cm}^{-1}$) and expressed as nmol g^{-1} .

Superoxide ($O_2^{\cdot-}$) Ions

These were determined spectrophotometrically at 480 nm by measuring the oxidation of epinephrine to adrenochrome [20]. The reaction mixture contained 75 mM PO_4^{3-} buffer (pH 7.4) and 0.2 ml extract. The amount of $O_2^{\cdot-}$ generated was calculated using extinction coefficient of $4,020\text{ M}^{-1}\text{ cm}^{-1}$ and expressed as $\mu\text{M g}^{-1}$.

Hydroxyl (OH^{\cdot}) Ions

These were determined by incubating roots (50 mg) in PO_4^{3-} buffer (10 mM, pH 7.4) containing 2-deoxyribose (15 mM)

at $37^\circ C$ for 2 h [21]. Thereafter, 0.7 ml of the above solution was mixed with 3 ml of 0.5% TBA made in 5 mM sodium hydroxide (NaOH) and 1 ml glacial acetic acid. The reaction mixture was heated for 30 min at $100^\circ C$ and then allowed to cool at $4^\circ C$ for 10 min. The absorbance of the mixture was read at 532 and corrected for non-specific absorbance at 600 nm. OH^{\cdot} content was calculated using extinction coefficient $155\text{ mM}^{-1}\text{ cm}^{-1}$ and expressed as nmol g^{-1} .

Relative Electrolyte Leakage

Root tissue (~ 50 mg) was vibrated for 30 min in distilled water followed by the measurement of conductivity of bathing medium (C_1). The samples were boiled for 15 min and the final conductivity (C_2) of the bathing medium was measured. Percent REL was determined using the following formula:

$$\% \text{REL} = (C_1/C_2) \times 100$$

Cell Viability

This was determined using 2,3,5-triphenyl tetrazolium chloride (TTC) as per Steponkus and Lanphear [22]. Briefly, root tissue (50 mg) was incubated overnight in dark with 0.6% (w/v) TTC solution (2 ml). The red formazan formed traps the oxygen released through respiratory chain and thus provides a measure of cell viability. It was extracted in ethanol and the absorbance of the extract was measured at 530 nm and expressed with respect to control.

Antioxidant Enzymes Assay

Roots (250 mg) were homogenized in 10 ml pre-chilled PO_4^{3-} buffer (0.1 M, pH 7.0) under ice-cold conditions. The homogenate was filtered through three layers of cheesecloth and centrifuged at $15,000\times g$ for 30 min at $4^\circ C$. The supernatant was stored at $4^\circ C$ until used for assaying the activities of enzymes: SOD, CAT, APX, GPX, and GR. An aliquot (0.5 ml) of the supernatant was used for determining the protein content against a calibration standard of bovine serum albumin [15]. All enzyme assays were performed at $25^\circ C$ using a UV-VIS spectrophotometer (Model 1700; Shimadzu Corporation, Tokyo, Japan).

SOD activity was determined in terms of its ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) as per Beauchamp and Fridovich [23]. The reaction mixture consisted of 63 μM NBT, 13 mM methionine, 0.1 mM EDTA, 13 μM riboflavin, 0.05 M sodium carbonate and 0.5 ml enzyme extract (0.5 ml distilled water in the control). Test tubes were exposed to two 15-W fluorescent lamps for 20 min followed by incubation in the

dark for 20 min. The absorbance was read at 560 nm and activity expressed as enzyme units (EU) mg^{-1} protein. One EU was the amount of enzyme required to cause 50% inhibition of NBT reduction at 25°C. Activities of APX, GPX, CAT and GR were assayed as described by Singh et al. [11]. Briefly, APX was assayed in terms of decrease in absorbance at 290 nm due to oxidation of ascorbate to dehydroascorbate and activity was determined using $\epsilon=2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. CAT was measured at 240 nm using $\epsilon=39.4 \text{ mM}^{-1} \text{ cm}^{-1}$ in terms of the rate of disappearance of H_2O_2 . GPX was assayed by measuring the increased absorbance at 470 nm due to oxidation of guaiacol and calculated using $\epsilon=26.6 \text{ mM}^{-1} \text{ cm}^{-1}$. Activity of GR was assayed in terms of oxidation of NADPH at 340 and using $\epsilon=6.224 \text{ mM}^{-1} \text{ cm}^{-1}$. The activities of APX, GPX, CAT and GR were expressed as EU mg^{-1} protein, where 1 EU was the amount of enzyme that decomposes 1.0 μM of ascorbate or H_2O_2 or NADPH or guaiacol, respectively, min^{-1} at 25°C [9].

Statistical Analyses

The experiments were conducted in a CRD with ten independent Petri dish replicates each having ten seeds. All the experiments and analyses were repeated twice. Since the difference between the two set of experiments was less than 5%, the data from only one set is presented. All the biochemical analyses and enzyme assays involved five independent tissue replicates. The data were analysed by one-way ANOVA followed by the separation of means using post-hoc Tukey's test at $P \leq 0.05$.

Results

Effect of As on Radicle Elongation

As-exposure adversely affected the radicle emergence and elongation in *P. aureus* in a concentration- and time-

dependent manner (Table 1). After 12 h of exposure, the radicle length was reduced by ~14% in response to 5 μM As and declined further to 50% of the control at 10 μM . After 24 and 48 h of As treatment, radicle length declined over that in control by 42–65% and 24–78%, respectively (Table 1).

Effect on Protein and Carbohydrate Content

As exposure significantly enhanced (by 14–61%) the protein content in *P. aureus* radicles in a concentration- and time-dependent manner. Protein content increased by 40–61% over control during 12–48 h of exposure to 50 μM As (Table 1). In contrast, a significant reduction in total carbohydrate content was observed in mung bean radicles upon As-treatment in a dose- and exposure-dependent manner. Total carbohydrate content declined by ~12–31%, ~23–37% and ~26–44% over control after 12, 24 and 48 h, respectively, in response to As exposure (Table 1).

Effect on Protein- and Starch-Metabolizing Enzymes

Activity of proteases decreased significantly in response to As treatment. After 24 h exposure to 50 μM As, it was reduced by 29–38% of that in control. It declined further with time period and after 48 h of treatment it was 47–53% of the control (Table 2). Activity of α - and β -amylases, starch phosphorylases, and acid invertases increased in response to As exposure. In response to 50 μM As, after 12 h the activity of α -amylases was ~2 times of that in the control. It enhanced further and was nearly 3 times after 24 and 48 h of exposure (Table 2). Likewise, the activity of β -amylases was 2–2.6 times of the control in response to 50 μM . In contrast, the activity of starch phosphorylases enhanced significantly only at $\geq 10 \mu\text{M}$ As. It was ~3.5, 4.3 and 4.8 times of the control after 12, 24 and 48 h, respectively, of exposure to 50 μM As (Table 2). As-treatment (5–50 μM) enhanced acid invertase activity over that in control by

Table 1 Effect of arsenic (As) on radicle emergence, and the contents of total protein and carbohydrate in the radicles of *P. aureus* when measured 12, 24 and 48 h after treatment

As (μM)	Radicle length (cm) ¹			Protein content (mg g^{-1})			Total carbohydrate content (mg g^{-1})		
	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h
0.0	0.22a	0.52a	0.91a	28.6a	29.7a	30.1a	22.8a	20.3a	18.6a
5.0	0.19a (-13.6) ²	0.30b (-42.3)	0.69b (-24.2)	32.7b (+14.3)	34.3b (+15.5)	35.9b (+19.3)	20.0b (-12.3)	15.7b (-22.7)	13.8b (-25.8)
10.0	0.11b (-50.0)	0.22c (-57.7)	0.38c (-58.2)	36.4c (+27.3)	37.25c (+25.6)	40.2c (-33.6)	16.8c (-26.3)	13.9c (-31.5)	11.5c (-38.2)
50.0	nm	0.18c (-65.4)	0.20d (-78.0)	39.9d (+39.5)	42.3d (+42.4)	48.3d (-60.5)	15.7d (-31.1)	12.7d (-37.4)	10.4d (-44.1)

¹ Means with different letters in a column are significantly different at $P \leq 0.05$ according to Tukey's test

² Figures in parenthesis represent percent change (+, increase; -, decrease) over the control

nm not measurable

Table 2 Effect of arsenic (As) on the activities of protein- and starch-hydrolyzing enzymes (α - and β -amylases, starch phosphorylases and acid invertases) in the radicles of *P. aureus* when measured 12, 24 and 48 h after treatment

As (μ M)	Proteases (μ g h ⁻¹ mg ⁻¹ protein)			α -Amylases (μ g min ⁻¹ mg ⁻¹ protein)			β -amylases (μ g min ⁻¹ mg ⁻¹ protein)			Starch phosphorylases (μ mol Pi s ⁻¹ mg ⁻¹ protein)			Acid invertases (μ g min ⁻¹ mg ⁻¹ protein)		
	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h
0.0	9.7±0.07a	11.9±0.12a	14.1±0.21a	5.1±0.07a	7.0±0.17a	10.3±0.11a	10.0±0.17a	13.1±0.19a	14.6±0.97a	0.2±0.03a	0.3±0.02a	0.5±0.02a	24.3±1.03a	29.7±0.84a	34.1±1.08a
5.0	7.9±0.10b	8.8±0.13b	11.5±0.18b	6.3±0.11b	10.6±0.06b	15.9±0.44b	12.0±0.12b	18.5±0.75b	18.1±0.45b	0.3±0.01a	0.7±0.04b	0.8±0.01b	26.7±0.87ab	33.5±0.49a	40.8±1.46b
10.0	6.9±0.16c	7.5±0.09c	8.7±0.16c	7.1±0.12c	15.7±0.15c	22.1±0.06c	16.6±0.31c	23.5±0.18bc	26.3±0.24c	0.5±0.02b	0.9±0.03c	1.5±0.03c	30.9±1.49b	38.6±1.27b	49.6±1.23c
50.0	5.1±0.12d	5.9±0.16d	6.6±0.13c	10.5±0.14d	20.9±0.10d	28.7±0.34c	19.5±0.29d	27.7±0.12c	38.6±0.22d	0.7±0.08c	1.3±0.02cd	2.4±0.04d	34.7±1.38c	44.3±2.01c	58.7±2.01d

Data presented as mean \pm SE; means with different letters in a column are significantly different at $P \leq 0.05$ according to Tukey's test

9.9–42.8%, 12.8–49.2% and 19.6–72.1% after 12, 24 and 48 h, respectively (Table 2).

Effect on ROS Generation

As exposure significantly enhanced MDA and H₂O₂ content in emerging radicles of *P. aureus*. MDA content increased over control by 46–131%, 42–112%, and 52–252% after 12, 24 and 48 h of exposure to 5–50 μ M As (Table 3). Likewise, H₂O₂ content increased by 23–82%, 28–117% and 64–216% after 12, 24 and 48 h of As (5–50 μ M) exposure over that in control radicles (Table 4).

O₂^{-•} content increased significantly ($P \leq 0.05$; except at 5 μ M As after 12 h) in *P. aureus* radicles upon exposure to As (Table 3). In response to 10 μ M As, O₂^{-•} increased over the control by 15–28%, whereas at 50 μ M As ~62% and 104% increase was noticed after 12 and 48 h exposure (Table 3). Likewise, the content of OH[•] increased significantly in *P. aureus* radicles in response to As. It increased over the control by ~17–92%, 20–213% and 50–278% at 12, 24 and 48 h, respectively, after exposure to 5–50 μ M As (Table 3).

Effect on Membrane Integrity and Cell Viability

As exposure enhanced REL in *P. aureus* radicles, thereby suggesting membrane disruption. REL enhanced over the control by ~8–34%, 13–39%, and 18–49% after 12, 24, and 48 h of exposure to As (Table 4). Furthermore, there was a decline in the cell viability in *P. aureus* radicles in response to As (5–50 μ M). The cell viability declined by ~9–26%, 17–32%, and 21–43% after 12, 24 and 48 h, respectively, of exposure to 5–50 μ M As (Table 4).

As-Induced Activities of Antioxidant Enzymes

After 12 h exposure, SOD activity enhanced by ~10% and 39% at 5 and 50 μ M As over that in the control. It enhanced further and after 24 and 48 h of As exposure, it was ~12–43% and 54–77% greater over that in the control (Fig. 1a). Unlike SOD, the CAT activity was not affected with time of exposure though it enhanced with increasing As concentrations (Fig. 1b). At 50 μ M As, CAT activity was ~70% higher than that in the control. APX activity enhanced over the control by 8–80%, 12–89% and 13–116% after 12, 24 and 48 h exposure to 5–50 μ M As (Fig. 1c). Activity of GPX and GR also increased in response to As exposure; however, the level of increase was lesser in GPX than in GR. GPX activity enhanced by 9–18%, 8–23%, and 7–43% after 12, 24 and 48 h, respectively, of As exposure (Fig. 1d). GR activity enhanced by 43–60%, 75–80%, and 83–120% in response to 5, 10 and 50 μ M As, respectively (Fig. 1e).

Table 3 Effect of arsenic (As) on malondialdehyde (MDA) and hydrogen peroxide (H₂O₂), superoxide anion (O₂⁻) and hydroxyl radical (OH[•]) content in the radicles of *P. aureus* when measured 12, 24 and 48 h after treatment

As (μM)	MDA (n mol g ⁻¹) ¹			H ₂ O ₂ (n mol g ⁻¹)			O ₂ ⁻ content (μM g ⁻¹)			OH [•] (n mol g ⁻¹)		
	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h	12 h	24 h	48 h
0.0	8.4±0.59a	10.8±0.67a	12.1±0.87a	18.5±1.34a	20.9±1.83a	22.1±1.87a	2.6±0.02a	2.7±0.02a	2.9±0.03a	1.2±0.14a	1.5±0.13a	1.8±0.16a
5.0	12.3±1.12b (46.4) ²	15.3±1.10b (41.7)	18.4±1.09b (52.1)	22.8±1.45b (23.2)	26.7±1.56b (27.8)	36.2±2.69b (63.8)	2.7±0.02a (3.8)	2.9±0.03b (7.4)	3.2±0.04b (10.3)	1.4±0.10a (16.7)	1.8±0.27a (20.0)	2.7±0.22b (50.0)
10.0	16.3±1.19c (94.0)	19.4±1.06c (79.6)	33.9±1.20c (180.2)	29.1±2.18c (57.3)	38.8±2.97c (85.6)	54.6±2.89c (147.1)	3.0±0.03b (15.4)	3.3±0.04c (22.2)	3.7±0.04c (27.6)	1.9±0.11b (58.3)	2.8±0.26b (86.7)	3.4±0.21c (88.9)
50.0	19.4±2.09c (130.9)	22.9±1.34d (112.0)	42.6±1.47d (252.1)	33.6±3.09c (81.6)	45.3±4.06c (116.7)	69.9±4.14d (216.3)	4.2±0.04c (61.5)	4.8±0.06c (77.8)	5.9±0.07d (103.5)	2.3±0.10c (91.7)	4.7±0.37c (213.3)	6.8±0.49d (277.8)

¹ Data presented as mean ± SE; means with different letters in a column are significantly different at P≤0.05 according to Tukey's test

² Figures in parenthesis represent percent increase over the control

Discussion

This study showed that As-inhibited radicle emergence and growth in germinating seeds of *P. aureus*. It was accompanied by alterations in the activities of sugar-metabolizing enzymes and oxidative damage in a time- and concentration-dependent manner. Though earlier studies have reported that As-exposure inhibits root growth [7–9], yet not much is known about the effect of As on sugar metabolism during germination. We observed that As-exposure increased the protein content and decreased carbohydrate content. In the literature, there are different opinions on the alterations in protein and starch content in the presence of heavy metals. Earlier studies have reported increased protein content in response to As [24] and Ni [25] in rice, to Mo in *Beta vulgaris* [26], and to Pb in *Brassica campestris* [27]. In contrast, a reduction in protein content has been reported in response to Ni and Cd in *B. vulgaris* [26] and to Cu in barley [28]. The enhanced protein content was accompanied by decreased proteases activity in emerging radicles. It corroborated with previous studies reporting downregulation of proteases in response to As [24], Ni [25], and Pb [27]. In our study, we observed an increase in the activity of starch-degrading enzymes (α- and β-amylases and starch phosphorylases) and sucrose-cleaving enzymes (acid invertases). These observations are in sharp contrast to earlier studies reporting decreased activity of α-amylases in response to Pb [27] and Cu [29] and of acid invertases in response to Pb [27], Cu [29] and Ag [30]. Nevertheless, an increase in the activity of acid invertases in response to Cd has been reported in rice [31]. α-Amylases and starch phosphorylases are the key enzymes of starch hydrolysis, whereas acid invertases are responsible for the generation of glucose and fructose (hexoses) from sucrose. A direct correlation exists between the activity of acid invertases and the hexoses [32]. Sucrose is a major carbon source in growing seedlings. Enhanced activity of acid invertases in response to As suggests greater degradation of sucrose, thereby limiting its availability for growing radicles. In other words, As toxicity favours greater hexose formation. Acid invertases play an important role in loading/uploading of phloem by maintaining sucrose concentration gradient [32]. Enhanced activity of sucrose-degrading enzymes suggests greater accumulation of hexose, which are involved in maintaining osmotic pressure and providing protection to biomolecules and biomembranes [33] and scavenge ROS [34]. Hexoses play a significant role in providing energy/carbon blocks for growing seedlings. Studies have demonstrated that activity of acid invertases is enhanced in metal-tolerant genotypes and it helps in providing metal tolerance to plants by greater expression of acid-invertase genes [35]. Sugars have been suggested to be involved in response to stresses and form intricate

Table 4 Effect of arsenic (As) on relative electrolyte leakage (REL) and cell viability in the radicles of *P. aureus* when measured 12, 24 and 48 h after treatment

As (μM)	REL (%) ¹			Cell viability (% w.r.t. control)		
	12 h	24 h	48 h	12 h	24 h	48 h
0.0	16.1 \pm 0.81a (0) ²	17.6 \pm 0.72a (0)	18.3 \pm 0.83a (0)	100 \pm 0.0a (0)	100 \pm 0.0a (0)	100 \pm 0.0a (0)
5.0	17.4 \pm 1.01a (+8.1)	19.8 \pm 1.19a (+12.5)	21.5 \pm 0.64a (+17.5)	90.7 \pm 2.05b (-9.3)	82.7 \pm 1.14b (-17.3)	79.2 \pm 1.23b (-20.8)
10.0	18.6 \pm 0.78a (+15.5)	21.9 \pm 0.89b (+24.4)	24.8 \pm 1.27b (+35.5)	84.7 \pm 1.11b (-15.3)	71.3 \pm 1.48c (-28.7)	67.4 \pm 1.76c (-32.6)
50.0	21.7 \pm 1.34b (+34.1)	24.4 \pm 1.49c (+38.6)	27.2 \pm 1.39c (+48.6)	73.8 \pm 2.13c (-26.2)	67.8 \pm 2.51c (-32.2)	57.2 \pm 1.63d (-42.8)

¹ Data presented as mean \pm SE; means with different letters in a column are significantly different at $P \leq 0.05$ according to Tukey's test

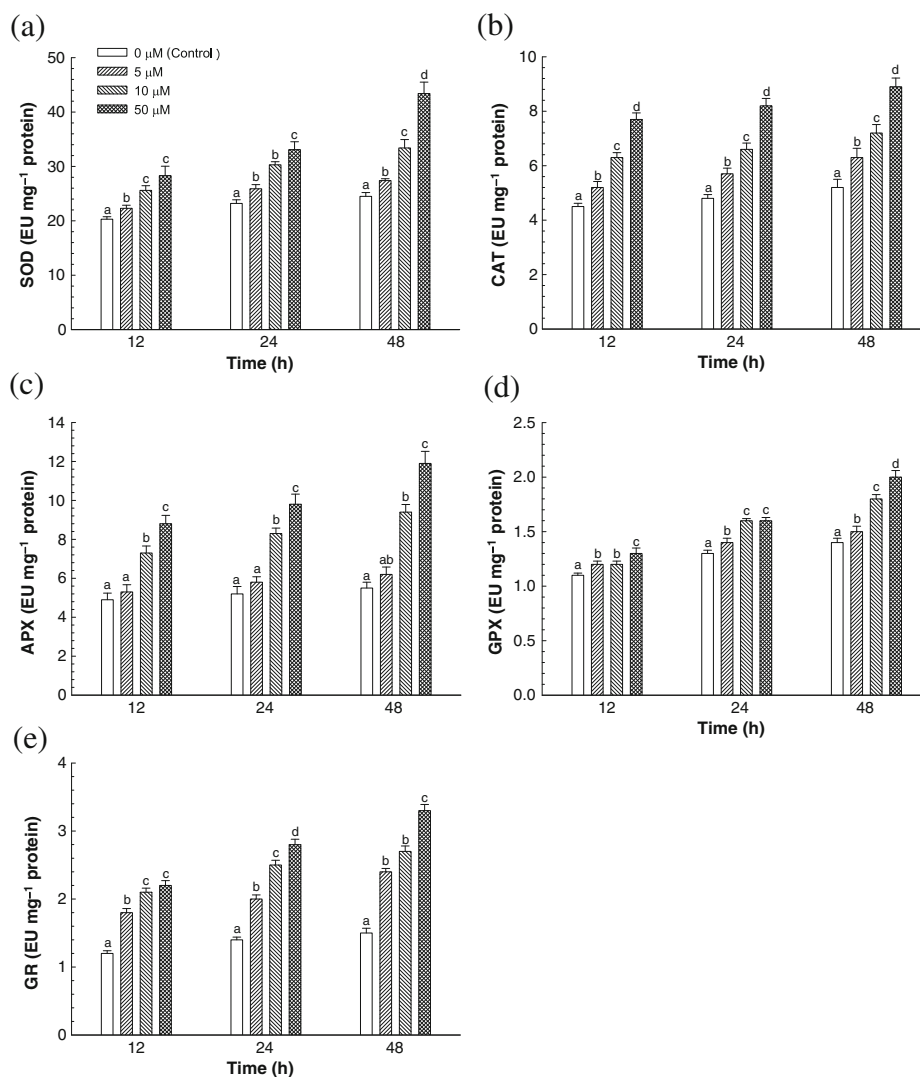
² Figures in parenthesis represent percent change (+, increase; -, decrease) over the control

network with ROS, quenching ROS and help in stress tolerance [36]. The decrease in soluble sugars has been correlated to the stimulation of respiration rate [37]. As exposure reduced cellular viability in emerging radicles of *P. aureus*. Arsenate is a well known de-coupler of phosphorylation in mitochondria and causes cellular death

[38]. Inside cytoplasm, being an analogue to phosphate, As competes with phosphate in ATP to form unstable product ADP-As resulting in disruption of energy metabolism in cells [39].

In the present study, MDA content, an indicator of lipid peroxidation, was enhanced in As-treated *P. aureus*, thereby

Fig. 1 Effect of Arsenic (As) on the activities of **a** SOD, **b** CAT, **c** APX, **d** GPX and **e** GR in the radicles of *P. aureus* measured 12, 24, and 48 h after treatment. Data presented as mean \pm SE. Different letters within a particular stage (12, 24, or 48 h) represent significant difference among them at $P \leq 0.05$ applying post-hoc Tukey's test



suggesting As-induced oxidative damage. As-induced ROS-mediated oxidative damage was further confirmed by enhanced production of H_2O_2 , O_2^- and OH^\bullet in *P. aureus* radicles. ROS are strong oxidizing agents causing oxidative damage to metabolically important biomolecules [40], which further leads to enhanced lipid peroxidation [41]. Lipid peroxidation result in reduced fluidity of membranes, increase the leakiness of membrane to substances that do not cross it, damage membrane proteins, and inactivates receptors, enzymes and ion channels [42]. Generation of O_2^- further triggers production of more reactive ROS such as OH^\bullet , which damage biological membranes. Loss of membrane integrity was confirmed by enhanced REL from radicles of As-exposed *P. aureus*. It is in agreement to earlier studies demonstrating lipid peroxidation in response to As [7–9]. Plants have evolved well-organized and intricate antioxidant mechanism (enzymatic and non-enzymatic) to scavenge free radicals to protect cells and subcellular systems [40]. In our study, activities of antioxidant enzymes (SOD, CAT, APX, GR, and GPX) were increased in As-treated *P. aureus* radicles. Earlier, As exposure reduced the activity of APX and CAT in 1-week-old *P. aureus* seedlings [9]. The increased activities of SOD in *P. aureus* radicles may be due to de novo synthesis of enzymatic proteins. Earlier, Ruiz-Lozano et al. [43] reported increased SOD activity in arbuscular mycorrhizal lettuce plants under drought stress and suggested it to be de novo synthesis of enzymes. Hartley-Whitaker et al. [7] demonstrated that SOD activity was enhanced upon exposure to both arsenate and arsenite in *Zea mays* as well as in arsenate-tolerant *Holcus lanatus*. SOD catalyses O_2^\bullet ions into H_2O_2 , which are further scavenged by CAT or APX in ascorbate–glutathione cycle [44]. Despite the fact that H_2O_2 scavenging enzymatic activity (CAT and APX) was upregulated in As-treated radicles, the content of H_2O_2 was greater, thus suggesting that enhanced CAT and APX activities are insufficient to scavenge excess H_2O_2 . It further indicated peroxidation of membrane lipids through accumulation of H_2O_2 and hence the extent of oxidative stress upon As exposure. The activity of GPX and GR increased with increasing concentration of As, being the maximum at high As treatment after 48 h. Recent evidences have indicated that peroxidases alleviate oxidative stress induced by metalloids [11]. Activity of GPX increased in response to As treatment indicating accumulation of H_2O_2 and ROS in plant tissue, which further stimulated the biosynthesis of peroxidases. It is well known that GR activity upon abiotic stress play a vital role in recycling oxidized glutathione to reduced one and maintains ratio of oxidized glutathione and total glutathione pool (GSH/GSSG) [45]. Within the cell arsenate changes to arsenite that has great affinity for thiol and sulfhydryl ($\sim\text{SH}$) group, e.g., glutathione, and results in inhibition of cell functioning

and cell death [46]. Gajewska et al. [47] suggested that enhanced GPX co-relates to greater lignification and thus stunted growth.

In conclusion, our results showed that As-inhibited radicle emergence and elongation in germinating *P. aureus* seeds by altering sugar metabolism and inducing a ROS-mediated oxidative damage.

References

1. Mahimairaja S, Bolan NS, Adriano DC, Robinson B (2005) Arsenic contamination and its risk management in complex environmental settings. *Adv Agron* 86:1–82
2. Naidu R, Bhattacharya P (2009) Arsenic in the environment – risks and management strategies. *Environ Geochem Health* 31:1–8
3. Williams PN, Villada A, Deacon C, Raab A, Figuerola J, Green AJ, Feldmann J, Meharg AA (2007) Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain levels compared to wheat and barley. *Environ Sci Technol* 41:6854–6859
4. Polizzotto M, Kocar BD, Benner SG, Sampson M, Fendorf S (2008) Near surface wetland sediments as a source of arsenic release to ground water in Asia. *Nature* 454:505–508
5. Lou-Hing D, Zhang B, Price AH, Meharg AA (2011) Effects of phosphate on arsenate and arsenite sensitivity in two rice (*Oryza sativa* L.) cultivars of different sensitivity. *Environ Exp Bot* 72:47–52
6. Smith E, Naidu R, Alston AM (1998) Arsenic in the soil environment: a review. *Adv Agron* 64:149–195
7. Hartley-Whitaker J, Ainsworth G, Meharg AA (2001) Copper and arsenate induced oxidative stress in *Holcus lanatus* L. clones with differential sensitivity. *Plant Cell Environ* 24:13–22
8. Stoeva N, Berova M, Zlatev Z (2005) Effect of arsenic on some physiological parameters in bean plants. *Biol Plant* 49:293–296
9. Singh HP, Batish DR, Kohli RK, Arora K (2007) Arsenic-induced root growth inhibition in mung bean (*Phaseolus aureus* Roxb.) is due to oxidative stress resulting from enhanced lipid peroxidation. *Plant Growth Regul* 53:65–73
10. Meharg AA (2003) Variation in arsenic accumulation—hyperaccumulation in ferns and their allies. *New Phytol* 157:25–31
11. Singh HP, Kaur S, Batish DR, Sharma VP, Sharma N, Kohli RK (2009) Nitric oxide alleviates arsenic toxicity by reducing oxidative damage in the roots of *Oryza sativa* (rice). *Nitric Oxide* 20:289–297
12. Ma B, Wan J, Shen Z (2007) H_2O_2 production and antioxidant responses in seeds and early seedlings of two different rice varieties exposed to aluminum. *Plant Growth Regul* 52:91–100
13. Van den Broeck K, Vendecasteele C, Geuns JMC (1998) Speciation by liquid chromatography-inductively coupled plasma-mass spectrometry of arsenic in mung bean seedlings used as a bio-indicator for the arsenic contamination. *Anal Chim Acta* 361:101–111
14. Sheppard SC (1992) Summary of phytotoxic levels of soil arsenic. *Water Air Soil Pollut* 64:539–550
15. Lowry OH, Rosebrough NT, Farr AL, Randall RJ (1951) Protein measurement with the folin–phenol reagent. *J Biol Chem* 193:265–275
16. Batish DR, Singh HP, Setia N, Kaur S, Kohli RK (2006) Effect of 2-benzoxazolinone (BOA) on seedling growth and associated biochemical changes in mung bean (*Phaseolus aureus*). *Z Naturforsch C* 61:709–714
17. Fiske CH, Subbarow Y (1952) The colorimetric determination of phosphorus. *J Biol Chem* 56:375
18. Nelson N (1994) A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem* 153:375–380

19. Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125:189–198
20. Misra HR, Fridovich I (1972) The univalent reduction of oxygen by reduced flavins and quinines. *J Biol Chem* 247:188–192
21. Halliwell B, Gutteridge JMC, Auroa O (1987) The deoxyribose method: a simple 'test tube' assay for determination of rate constants for reactions of hydroxyl radicals. *Ann Biochem* 165:215–219
22. Steponkus PL, Lanphear FR (1967) Refinement of triphenyl tetrazolium chloride method of determining cold injury. *Plant Physiol* 42:1423–1426
23. Beauchamp CO, Fridovich I (1971) Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* 44:276–287
24. Mishra S, Dubey RS (2006) Inhibition of ribonuclease and proteases activities in arsenic exposed rice seedlings: role of proline as enzyme protectant. *J Plant Physiol* 163:927–936
25. Maheshwari R, Dubey RS (2007) Nickel toxicity inhibits ribonuclease and proteases activities in rice seedlings: protective effects of proline. *Plant Growth Regul* 51:231–243
26. Kevresan S, Petrovic N, Popovic M, Kandrak J (1998) Effect of heavy metals on nitrate and protein metabolism in sugar beet. *Biol Plant* 41:235–240
27. Singh HP, Kaur G, Batish DR, Kohli RK (2011) Lead (Pb)-inhibited radicle emergence in *Brassica campestris* involves alterations in starch-metabolizing enzymes. *Biol Trace Elem Res*. doi:10.1007/s12011-011-9129-3
28. Demirevska-Kepova K, Simova-Stoilova L, Stoyanova Z, Hölzer R, Feller U (2003) Biochemical changes in barley plants after excessive supply of copper and manganese. *Environ Exp Bot* 52:253–266
29. Xiong ZT, Wang T, Liu K, Zhang ZZ, Gan JH, Huang Y, Li MJ (2008) Differential invertase activity and root growth between Cu-tolerant and non-tolerant populations in *Kummerowia stipulacea* under Cu stress and nutrient deficiency. *Environ Exp Bot* 62:17–27
30. Sturm A (1999) Invertases. Primary structures, functions, and roles in plant development and sucrose partitioning. *Plant Physiol* 121:1–7
31. Verma S, Dubey RS (2001) Effect of cadmium on soluble sugars and enzymes of their metabolism in rice. *Biol Plant* 44:117–123
32. Roitsch T, Gonzalez MC (2004) Function and regulation of plant invertases: sweet sensations. *Trends Plant Sci* 9:606–613
33. Sinniah UR, Ellis RH, John P (1998) Irrigation and seed quality development in rapid recycling *Brassica*, soluble carbohydrates and heat stable protein. *Ann Bot* 82:647–655
34. Deryabin AN, Siñkevich MS, Dubinina IM, Burakhanova EA, Trunova TI (2007) Effect of sugars on the development of oxidative stress induced by hypothermia in potato plants expressing yeast invertase gene. *Russ J Plant Physiol* 54:32–38
35. Huang W-X, Cao Y, Huang L-J, Ren C, Xiong ZT (2011) Differential expression of acid invertase genes in roots of metallophilous and non-metallophilous populations of *Rumex japonicus* under copper stress. *Chemosphere* 84:1432–1439
36. Bolouri-Moghaddam MR, Le Roy K, Xiang L, Rolland F, Van den Ende W (2010) Sugar signalling and antioxidant network connections in plant cells. *FEBS J* 277:2022–2037
37. Saleh M, Al-Garni S (2006) Increased heavy metal tolerance of cowpea plants by dual inoculation of an arbuscular mycorrhizal fungi and nitrogen-fixer *Rhizobium* bacterium. *Afr J Biotechnol* 5:133–142
38. Terwelle HF, Slater EC (1967) Uncoupling of respiratory chain phosphorylation by arsenate. *Biochim Biophys Acta* 143:1–17
39. Meharg AA (1994) Integrated tolerance mechanisms: constitutive and adaptive plant responses to elevated metal concentrations in the environment. *Plant Cell Environ* 17:989–993
40. Gunes A, Inal A, Bagci EG, Pilbeam DJ (2007) Silicon-mediated changes of some physiological and enzymatic parameters symptomatic for oxidative stress in spinach and tomato grown in sodic-B toxic soil. *Plant Soil* 290:103–114
41. Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7:405–410
42. Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiol Biochem* 48:909–930
43. Ruiz-Lozano JM, Azcon R, Palma JM (1996) Superoxide dismutase activity in arbuscular mycorrhizal *Lactuca sativa* plants subjected to drought stress. *New Phytol* 134:327–333
44. Cao X, Ma LQ, Tu C (2004) Antioxidant responses to arsenic in the arsenic-hyperaccumulator Chinese brake fern (*Pteris vittata* L.). *Environ Pollut* 128:317–325
45. Ekmekci Y, Tanyolac D, Ayhana B (2008) Effects of cadmium on antioxidant enzyme and photosynthetic activities in leaves of two maize cultivars. *J Plant Physiol* 165:600–611
46. Schmöger MEV, Oven M, Grill E (2000) Detoxification of arsenic by phytochelatins in plants. *Plant Physiol* 122:793–801
47. Gajewska E, Slaba M, Andrzejewska R, Sklodowska M (2006) Nickel-induced inhibition of wheat root growth is related to H₂O₂ production, but not to lipid peroxidation. *Plant Growth Regul* 49:95–103