

# Plant Growth and Leaf Antioxidant Metabolism of Four Elite Grass Pea (*Lathyrus sativus*) Genotypes, Differing in Arsenic Tolerance

Dibyendu Talukdar

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**Abstract** Response of four improved grass pea (*Lathyrus sativus* L.) genotypes to arsenic was tested in pot experiment using 30 mg As l<sup>-1</sup> up to 60 days of growth after commencement of treatment (DAC). Arsenic exposure significantly reduced growth and seed yield potential of vars B1 and BioL-212, but no such effect was observed in bold-seeded mutant and pod indehiscent mutant lines. Results revealed normal leaf photosynthesis and antioxidant metabolism at 20 DAC in both varieties. However, high superoxide dismutase activity coupled with low ascorbate redox and declining ascorbate peroxidase level led to abnormal rise in H<sub>2</sub>O<sub>2</sub> content at reproductive stages (40 and 60 DAC), consequently, resulting in significantly enhanced arsenic-induced oxidative damage and physiological impediment in both varieties. By contrast, H<sub>2</sub>O<sub>2</sub> level in both the mutants was remarkably modulated at reproductive stage (40 DAC) in a highly controlled manner by balanced action of antioxidant defense. This favored normal photosynthesis and ensured good grain yield even under prolonged arsenic exposure by effectively preventing oxidative damage to membrane in both the mutants. Native PAGE analysis revealed that enzyme isoforms were involved in the regulation of foliar H<sub>2</sub>O<sub>2</sub> metabolism, the level of which was found extremely critical in determining arsenic tolerance of grass pea genotypes.

**Keywords** Arsenic · Antioxidant defense · Oxidative stress · H<sub>2</sub>O<sub>2</sub> · Grain yield · Grass pea

## Introduction

Arsenic (As) is a ubiquitous toxic metalloid. In recent times, the impact of irrigation with high-arsenic-containing groundwater on soil and crop has drawn huge attention due to its transfer to the food chain via groundwater–plant–soil system [2, 12, 13]. The arsenate oxyanions HAsO<sub>4</sub><sup>2-</sup> and H<sub>2</sub>AsO<sub>4</sub><sup>-</sup> are the most prevalent forms of arsenic in surface soil, water, and within cells, and these oxyanions contain arsenic in the pentavalent state [As (V)]. Being chemically very similar to phosphate, arsenate [As (V)] is readily taken up by high-affinity phosphate transporter in plants and >95 % of it is readily reduced to more toxic arsenite [As (III)] *in planta* by arsenate reductase activity [1, 50]. While

As (III) is the predominant arsenic species in anaerobic (flooded paddy) soils, As (V) was prevalent in aerobic soils, and toxicity of arsenic often varies between crops depending on the nature of arsenic speciation [26, 50]. Flooding of paddy soils leads to mobilization of As (III) into the soil solution and transport of As (III) through highly efficient Si transport in rice [50]. At higher concentrations (>100 μM), As (III) influx was substantially faster than As (V), and unlike As (V) uptake, As (III) uptake was inhibited by glycerol and antimonite, but not by phosphate, and may be taken up by aquaporin channels such as different subfamilies of nodulin26-like intrinsic proteins (NIPs) and other intrinsic proteins involved in As (III) permeability through membrane in plant [7, 30]. In legume roots, the rhizobial bacteria enzymatically reduce absorbed arsenate to highly toxic arsenite and efflux the arsenite out of the cell through aquaglyceroporin channel [42]. In rice, bidirectional transport (influx and efflux) of As (III) is reportedly facilitated by silicon transporter Lsi1

D. Talukdar (✉)  
Department of Botany, R.P.M. College, University of Calcutta,  
Uttarpara, Hooghly 712258, West Bengal, India  
e-mail: dibyendutalukdar9@gmail.com

(OsNIP2;1, an aquaporin channel) [54]. However, besides its rapid efflux, As (III) is detoxified by complexation with either thiol-rich peptides such as reduced glutathione and phytochelatins (PCs) or vacuolar sequestration [50, 54] or a combination of both [50]. Recent study identified AtABCC1 and AtABCC2 as major vacuolar PC–arsenic transporters [39] in *Arabidopsis thaliana*, and the vacuolar transporter (ACR3) essential for As (III) transport and arsenic tolerance has been identified in arsenic hyperaccumulator fern, *Pteris vittata* [21]. Arsenate is efflux to the external medium similar to the phosphate efflux through anion channels [54].

Accumulating evidences indicates that arsenic can induce oxidative stress through excess reactive oxygen species (ROS) accumulation in major crops like cereals and legumes [15, 17, 18, 27, 35–37, 46]. As (V) exposure induces oxidative stress in rice seedlings [37], affecting amino acids, mineral nutrient status, antioxidant, phytohormone, and lipid metabolism [13, 53]. Differential response to As (III) and As (V) treatments was observed in rice transcriptomic analysis [10], during wheat seed germinations and carbohydrate metabolism [24], and in arsenic hyperaccumulator and sensitive plant species [40]. Among the legumes, arsenic reportedly perturbed photosynthetic capacity and impeded growth potential by inducing severe oxidative stress in pea, lentil, chickpea, mung beans, common beans, fenugreek and forage crops [28, 35, 36, 41, 46, 47]. To combat this stress, plants have developed well-integrated antioxidant defense systems, in which ascorbate (AsA) plays pivotal role as non-enzymatic antioxidant during scavenging of H<sub>2</sub>O<sub>2</sub>, a highly diffusible ROS [14]. Superoxide dismutase (SOD) constitutes the first line of defense against superoxide radicals, but it steadily generates H<sub>2</sub>O<sub>2</sub> during their dismutation [3], which is readily scavenged by AsA-dependent ascorbate peroxidase (APX) and by catalases (CAT) [14]. These three prominent H<sub>2</sub>O<sub>2</sub>-metabolizing enzymes hold key in controlling H<sub>2</sub>O<sub>2</sub> level during the onset of oxidative stress in plants [14]. Activities of all the three H<sub>2</sub>O<sub>2</sub>-metabolizing enzymes were decreased during the onset of arsenic-induced oxidative damage in *A. thaliana* [33], while their expressions were modulated in arsenic-treated root tips of *Zea mays* and *Vicia faba* [12]. Available evidence indicates differential responses of antioxidant defense between tolerant and sensitive genotypes of major cereals, legumes, tuber crops and vegetables [18, 34, 36, 46, 47].

Grass pea (*Lathyrus sativus* L.) is a widely cultivated hardy cool-season legume crop and is a promising source of seed protein, minerals and antioxidant compounds [44]. Preliminary reports indicated the inhibition of plant growth due to As accumulation [43]. Despite vast areas under pulse cultivation are metal/metalloid-contaminated, virtually nothing is known about growth response and intrinsic

antioxidant metabolism of grass pea to arsenic exposure. In order to ascertain the possible growth response, grain yield and role of H<sub>2</sub>O<sub>2</sub> metabolism in determining sensitivity to arsenic exposure, four improved grass pea genotypes were subjected to long-term arsenic treatment. Arsenic accumulation and yield parameters were studied at harvest, while leaf photosynthesis and antioxidant parameters were analyzed at three different time intervals of growth stage.

## Materials and Methods

### Plant Materials

Four grass pea genotypes, namely variety BioL-212 (Rattan) and B1 (Nirmal) developed through somaclonal variation and hybridization and two induced mutant lines, namely one bold-seeded mutant (BSM) and one pod indehiscent mutant (PIM), were developed through 350-Gy gamma-ray-treated progeny of grass pea var. BioR-231 were used in the present study. All the genotypes are high yielding and contain very low (<0.2 %) seed neurotoxin contents [43].

### Pot Experiment and Treatment Protocols

Fresh and healthy seeds of four grass pea genotypes (B1, BioL-212, BSM and PIM) were surface-sterilized with NaOCl (0.1 %, w/v) and continuously washed under running tap water followed by distilled water. Seeds were allowed to germinate in the dark in separate sets on moistened filter paper at 25 °C. Germinated seedlings were immediately transferred to 12-in. earthen pots containing a mixture of soil, vermiculite and farm yard manure (1:1:1), as described before [43, 45]. Seedlings were thinned to one per pot and watered evenly for uniform growth until 7 days after first emergence. The pots were kept under control condition (14-h photoperiod, 28/18 ± 2 °C, relative humidity of 70 ± 2 % and a photon flux density of 200 μmol m<sup>-2</sup> s<sup>-1</sup>.) in a randomized block design. Treatment was commenced on 15-day-old seedlings by watering the plants with arsenic solution (sodium arsenate, MW 312.01 g/mol; technical grade, purity 98.5 %, Sigma-Aldrich, Bangalore, India) of 30 mg l<sup>-1</sup> keeping a control set (distilled water instead of arsenic solution) for each genotype in four replicates treatment<sup>-1</sup>. A pilot experiment indicated significant effect of arsenic at this concentration on seed germination and early seedling growth of grass pea [43] and, thus, was selected for the present study. Control and treated plants were allowed to grow for another 60 days after commencement of treatment (DAC). Pot soil was refreshed thrice in a week to prevent the depletion of nutrients as well as arsenic in the course of the plant's

exposure to the metalloid. Water content of the soil was maintained at 80 % of water-holding capacity. At 60 DAC, shoots and roots were harvested separately and thoroughly washed with tap water, lengths were measured, and then dry weight was determined after drying shoots and roots at 70 °C for 48 h till constant weight. Plant growth and yield characteristics were recorded at harvest (60 DAC). Leaf biochemical characters and antioxidant metabolism were assessed in control and treated seedlings at 20 DAC (vegetative stage), 40 DAC (flowering stage) and 60 DAC (pod-bearing stage).

#### Measurement of Arsenic Content

The roots and shoots were digested in a  $\text{HNO}_3\text{--HClO}_4$  (3:1, v/v) mixture, and arsenic concentration was determined by flow injection hydride generation atomic absorption spectrophotometer (Perkin-Elmer, FIA-HAAS Analyst 400), keeping a Standard Reference Materials (SRMs) of tomato leaves (Item number 1573a, from National Institute of Standards and Technology, USA) as part of the quality assurance/quality control protocol, as described earlier [46, 47].

#### Determination of Chlorophyll and Total Carotenoids

Leaf chlorophyll (Chl) and carotenoid contents were determined by the method of Lichtenthaler [25]. The contents were expressed as mg chl or carotenoids  $\text{g}^{-1}$  fresh weight (FW). The chl stability indices (CSI %) were measured using the formula: (Total chl content in stressed leaves/total chl content in control leaves)  $\times$  100.

#### Photosynthetic Rate

Leaf photosynthetic rate was assayed following Coombs et al. [11] with a portable photosynthesis system (LI-6400XT, LI-COR, USA).

#### Determination of $\text{H}_2\text{O}_2$ Content

Fresh tissue of 0.1 g was powdered with liquid nitrogen and blended with 3 ml acetone for 30 min at 4 °C. Then, the sample was filtered through eight layers of gauze cloth. After the addition of 0.15 g of active carbon, the sample was centrifuged twice at 3,000g for 20 min at 4 °C, and then 0.2 ml 20 %  $\text{TiCl}_4$  in HCl and 0.2 ml ammonia were added to 1 ml of the supernatant. After reaction, the compound was centrifuged at 3,000g for 10 min, the supernatant was discarded, and the pellet was dissolved in 3 ml of 1 M  $\text{H}_2\text{SO}_4$ .  $\text{H}_2\text{O}_2$  content was measured from the

absorbance at 410 nm using a standard curve, following Wang et al. [51].

#### Estimation of Lipid Peroxidation and Electrolyte Leakage (EL)

Lipid peroxidation rate was determined by measuring the malondialdehyde (MDA) equivalents following Hodges et al. [19] and was expressed as  $\text{nmol MDA g}^{-1}$  FW. EL was assayed by measuring the ions leaching from tissue into deionized water [44].

#### Estimation of Foliar AsA

Reduced (AsA) and oxidized ascorbate (DHA) contents were determined following Law et al. [22]. AsA redox was calculated as  $\text{AsA}/(\text{AsA} + \text{DHA})$ .

#### Antioxidant Enzyme Assays

Fresh leaf tissue of 250 mg was homogenized in 1 ml of 50 mM K-phosphate buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol and 2 % (w/v) polyvinyl pyrrolidone (PVP) using a chilled mortar and pestle kept in an ice bath. The homogenate was centrifuged at 15,000g at 4 °C for 20 min. Clear supernatant was used for enzyme assays. For measuring APX activity, the tissue was separately ground in homogenizing medium containing 2.0 mM AsA in addition to the other ingredients. All assays were done at 25 °C. Soluble protein content was determined using BSA as a standard [8].

SOD (EC 1.15.1.1) activity was determined by nitro blue tetrazolium (NBT) photochemical assay [5] and was expressed as U (unit)  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein. One unit of SOD was equal to that amount causing a 50 % decrease in SOD-inhibited NBT reduction. SOD isozymes were individualized by native PAGE on 10 % acrylamide gel and were localized by a photochemical method [5]. Activity staining gels were incubated for 30 min in 50 mM K-phosphate buffer, pH 7.5, containing 2 mM KCN or 5 mM  $\text{H}_2\text{O}_2$ . Cu/Zn-SOD is inhibited by KCN and  $\text{H}_2\text{O}_2$ ; Fe-SOD is inactivated by  $\text{H}_2\text{O}_2$  but resistant to KCN, and Mn-SOD is resistant to both inhibitors. APX (EC 1.11.1.11) activity ( $\text{nmol AsA oxidized min}^{-1}$   $\text{mg}^{-1}$  protein) was assayed following Nakano and Asada [31] with  $\text{H}_2\text{O}_2$ -dependent oxidation of AsA followed by a decrease in the absorbance at 290 nm ( $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ). Native PAGE of APX isozymes was performed in 4 % gel and stained following Mittler and Zilinskas [29] based on the inhibition of NBT reduction by AsA. CAT (EC 1.11.1.6)-specific activity ( $\text{nmol H}_2\text{O}_2$  degraded  $\text{min}^{-1}$   $\text{mg}^{-1}$  protein) was calculated using the molar absorptivity of  $43.6 \text{ M}^{-1} \text{ cm}^{-1}$  for  $\text{H}_2\text{O}_2$  at

240 nm [9]. CAT isozyme profiling was done on 6 % acrylamide gel, following Woodbury et al. [52].

### Statistical Analyses

The results are the mean values  $\pm$  standard error (SE) of at least four replicates. Means were compared by ANOVA using the SPSS v. 10 (SPSS Inc., USA) and evaluated using Duncan's multiple range test at  $P \leq 0.05$ . Simple correlation was carried out among different traits using Microsoft Excel data analysis tool pack 2007, and the significance of correlation coefficient was tested (two-tailed mode) using the formula:  $t (t \text{ test}) = r \sqrt{(n - 2) / \sqrt{(1 - r^2)}}$ , where  $r$  = the sample correlation and  $n = 10$ , the sample size [42].

## Results

### Effect of Arsenic on Seedling Growth, Seed Yield and As Accumulation

Arsenic treatment ( $30 \text{ mg As l}^{-1}$ ) induced significant ( $P < 0.05$ ) variations among four grass pea genotypes for growth and seed yield components (Table 1). Comparing control, plant height, primary branches/plant, shoot dry weight, pods/plant, 100 seed weight and seed yield/plant were decreased significantly in variety B1 and BioL-212, but were quite normal in BSM and PIM mutant lines under arsenic exposure (Table 1). Root dry weight and length, however, were nearly normal in all treated genotypes. In general, roots contained higher arsenic in comparison with shoots, but substantial amount of arsenic was also measured in shoots of all genotypes with highest content in variety B1 and the lowest one in the BSM line (Table 1).

### Leaf Photosynthesis

In treated B1 and BioL-212, pigment composition, chl *a/b* ratio, CSI % and photosynthetic rate were marginally changed at 20 and 40 DAC, respectively, but were significantly reduced thereafter (Fig. 1a–c). All the parameters, however, were significantly lower in both BSM and PIM lines at 20 DAC, but became normal at 40 DAC and was stabilized at 60 DAC (Fig. 1a–c; Table 1). Considering all four genotypes, photosynthesis rate showed significant association with pods/plant ( $r = 0.82$ ,  $t = 4.052$ ), 100 seed weight ( $r = 0.70$ ,  $t = 2.772$ ), seed yield/plant ( $r = 0.75$ ,  $t = 3.207$ ) and shoot dry weight ( $r = 0.73$ ,  $t = 3.021$ ) at 60 DAC.

### Arsenic-induced $\text{H}_2\text{O}_2$ Accumulation, Lipid Peroxidation and Electrolyte Leakage (EL%)

Accumulation of  $\text{H}_2\text{O}_2$ , MDA and EL% was significantly higher in B1 and BioL-212 over control at 60 DAC, but was quite normal in B1 up to 20 DAC and in BioL-212 till 40 DAC (Fig. 1d–f). Contrastingly, arsenic treatment led to significant rise in  $\text{H}_2\text{O}_2$  level, MDA and EL% in BSM and PIM lines at 20 DAC, but barring  $\text{H}_2\text{O}_2$ , MDA content and EL% became normal at 40 and 60 DAC (Fig. 1d–f).

### Antioxidant Defense Responses to Arsenic Treatment

AsA content and AsA redox were significantly higher in arsenic-treated B1 at 20 DAC and BioL-212 up to 40 DAC, but was markedly reduced in both varieties at 60 DAC (Table 2). Both values were significantly lower in the mutant lines at 20 DAC, but were increased to normal level at 40 and 60 DAC (Table 2).

SOD activity was significantly higher over the control in BioL-212, BSM and PIM throughout the treatment period and in B1 at later stages of growth. APX activity was initially normal in B1 and BioL-212, but was reduced even below the control level at 60 DAC (Table 2). It was significantly low in mutant lines at 20 DAC, but was markedly enhanced over control at 60 DAC (Table 2). CAT activity was normal to high in B1 and both mutant lines at 60 DAC, but was significantly reduced in BioL-212 (Table 2).

Altogether, five activity bands were resolved for SOD on the basis of their increasing mobility and sensitivity to  $\text{H}_2\text{O}_2$  and KCN at 60 DAC (Fig. 2a, b). Cu/Zn I and II were consistently resolved in control plants (Fig. 2a). In addition to Cu/Zn I and II, Mn-SOD I was formed in B1 and BioL-212, exhibiting stronger intensity in case of latter. Mn-SOD I and II, and Mn-SOD I and Fe-SOD, additional to Cu/Zn-SOD I and II, were resolved in BSM and PIM, respectively (Fig. 2a, b). For APX, three isoforms (APX 1, APX 2 and APX 3) occurred in leaf extracts of control plants and in arsenic-treated B1 and BioL-212 at 60 DAC (Fig. 3a). Band intensity of all three isoforms was reduced considerably in B1 and BioL-212, but a new band resolved as APX 4 in addition to APX 1, APX 2 and APX 3 appeared in BSM and PIM lines (Fig. 3a). CAT activity was uniformly visualized as a single zone across the genotypes. Comparing control, band intensity was much higher in the two mutant lines, but was considerably lower in BioL-212 at 60 DAC (Fig. 3b).

## Discussion

Growth inhibition is the most distinct manifestation of arsenic-induced toxicity in plants [2, 12, 17, 43]. Arsenic

**Table 1** Effect of arsenic (As) treatments (30 mg l<sup>-1</sup>) on plant growth, yield and As accumulation in control and four treated genotypes of *L. sativus* L.

Traits	Control	As-treated genotypes			
		B1	BioL-212	BSM	PIM
Plant height (cm)	48.09 ± 1.47a	29.68 ± 2.39c	37.12 ± 1.52b	49.17 ± 1.49a	45.54 ± 1.18a
Primary branches/plant	12.15 ± 0.78a	5.50 ± 0.39b	7.90 ± 0.51c	13.65 ± 0.81a	12.95 ± 0.89a
Root length (cm)	16.38 ± 2.01a	16.13 ± 1.19a	17.10 ± 1.23a	17.21 ± 1.43a	16.23 ± 1.39a
Root DW (g/plant)	0.29 ± 0.80a	0.28 ± 0.91a	0.31 ± 0.29a	0.32 ± 0.71b	0.30 ± 0.87c
Shoot DW (g/plant)	0.19 ± 0.47a	0.05 ± 0.53b	0.06 ± 0.45b	0.22 ± 0.49a	0.20 ± 0.33a
Pods/plant	88.10 ± 3.84a	14.70 ± 4.35c	21.42 ± 4.11b	89.86 ± 0.39a	86.77 ± 0.42a
100 seed weight (g)	5.51 ± 1.28b	2.73 ± 1.30c	2.77 ± 1.36c	11.61 ± 1.18a	5.89 ± 1.19b
Seed yield/plant (g)	16.56 ± 5.20b	6.56 ± 0.33d	10.68 ± 0.89c	20.02 ± 3.46a	15.78 ± 3.69b
As content (mg kg <sup>-1</sup> DW)					
Shoots	0.02 ± 0.16d	0.51 ± 0.21a	0.39 ± 0.30b	0.15 ± 0.48c	0.18 ± 0.18c
Roots	0.03 ± 0.18c	0.60 ± 0.22a	0.53 ± 0.43a	0.19 ± 0.36b	0.22 ± 0.44b
Chl <i>a</i> (mg g <sup>-1</sup> FW)	4.11 ± 0.38a	1.03 ± 0.49c	1.37 ± 0.39b	4.13 ± 0.39a	4.09 ± 0.40a
Chl <i>b</i> (mg g <sup>-1</sup> FW)	1.79 ± 0.37a	1.81 ± 0.42a	1.75 ± 0.37a	1.77 ± 0.39a	1.71 ± 0.40a
Chl <i>a/b</i>	2.30 ± 0.31a	0.57 ± 0.41a	0.78 ± 0.47b	2.33 ± 0.33c	2.61 ± 0.40d
CSI %	100.0 ± 0.23a	48.15 ± 0.38a	52.90 ± 0.28a	100.0 ± 0.25a	98.0 ± 0.33a
Carotenoids (mg g <sup>-1</sup> FW)	1.51 ± 0.31a	1.49 ± 0.30a	1.51 ± 0.30a	1.49 ± 0.29a	1.50 ± 0.35a
Leaf photosynthetic rate, μM CO <sub>2</sub> /(m <sup>2</sup> s)	13.19 ± 0.76a	6.39 ± 0.49d	7.11 ± 0.53c	12.76 ± 0.66b	13.45 ± 0.81a

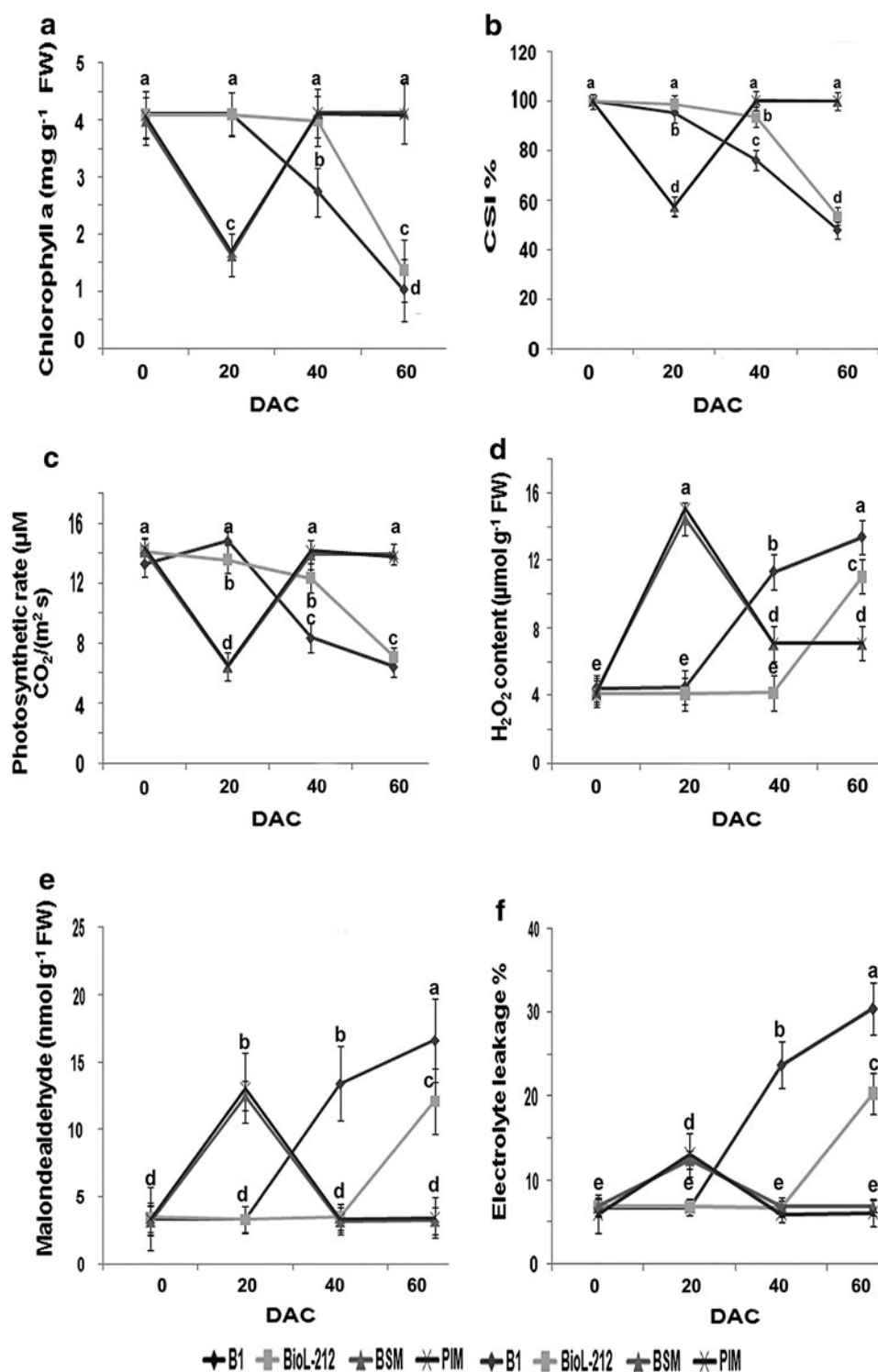
Data were recorded at harvest (60 DAC) and presented as mean ± SE of four replicates. Different letters in each row indicate significant differences at  $P \leq 0.05$  in ANOVA followed by Duncan's multiple range test

effect at 30 mg l<sup>-1</sup> on growth of 60-day-old treated grass pea genotypes observed in the present investigation, however, is not straightforward, and significant variation was observed among four genotypes for the traits. Decreased shoot dry weight in B1 and BioL-212 under arsenic treatment might be attributed to significant reduction in plant height ( $r = 0.72$ ,  $t = 2.935$ ), number of primary branches ( $r = 0.82$ ,  $t = 4.052$ ), and to low pods/plant ( $r = 0.82$ ,  $t = 4.052$ ) and 100 seed weight ( $r = 0.73$ ,  $t = 2.778$ ). Significant positive correlation of photosynthesis with shoot dry weight and yield components at 60 DAC suggested serious consequences of low photosynthesis at reproductive stage on grain yield of the treated varieties. By contrast, despite the disruption of photosynthetic apparatus at initial growth period, good grain yield in the treated mutants was ensured through efficient and timely turnover of photosynthesis at reproductive stages (40, 60 DAC) when mobilization of food materials toward sink is an important event for proper grain filling [16]. The restoration of normal photosynthesis in the two mutants despite continuous arsenic exposure has immense agronomic significance as photosynthetic pigments are some of the most important internal physiological factors, which are believed to be the targets of arsenic-induced toxicity in crop plants [35, 36, 41, 46, 47]. However, shoot/root arsenic in the present control plants (0.67) increased to 0.85 in B1, 0.74 in BioL-212, 0.79 in BSM and 0.82 in PIM,

strongly indicating arsenic accumulation in shoots of treated seedlings, and agreed well with reported bioaccumulation and transport of arsenic in vegetative and edible part of major grain, spice and forage legumes [17, 35, 46, 47].

In order to understand the intrigues behind differential arsenic sensitivity of four genotypes, role of AsA and activities of three prominent H<sub>2</sub>O<sub>2</sub>-metabolizing enzymes were assayed. These four components play vital roles in primary defense mechanism of plants during ROS detoxification particularly H<sub>2</sub>O<sub>2</sub> [4, 14]. The dual roles of H<sub>2</sub>O<sub>2</sub> as a stress inducer and as a signaling molecule to upregulate antioxidant defense against oxidative damage have been increasingly recognized [20, 32, 45]. In the present study, a time-bound measurement at 20, 40 and 60 DAC revealed a completely reverse situation between varieties and mutant lines for H<sub>2</sub>O<sub>2</sub> level. High SOD activity strongly suggested arsenic-induced generation of excess superoxide radicals, and subsequently, their dismutation formed huge H<sub>2</sub>O<sub>2</sub> in treated seedlings. Results indicated that the normal level of leaf H<sub>2</sub>O<sub>2</sub> in the treated varieties was maintained by opposite mechanisms of SOD and APX, balancing it to a particular level during initial treatment period. Despite normal CAT activity, this critical balance, however, was lost when APX activity began to decline in both the varieties at reproductive period due to prolonged arsenic exposure. Same situation was experienced by

**Fig. 1** Changes in **a** chlorophyll *a* content, **b** chlorophyll stability index (CSI %), **c** photosynthetic rate, **d** accumulation of H<sub>2</sub>O<sub>2</sub>, **e** malondialdehyde content and **f** electrolyte leakage % in leaves of variety B1 and BioL-212 and BSM—bold-seeded mutant and PIM—pod indehiscent mutant of grass pea at 20, 40 and 60 days after commencement (DAC) of arsenic treatment (0) with sodium arsenate at 30 mg l<sup>-1</sup>. Data presented here are mean ± SE of four replicates. Means with different small letters are significantly different at *P* ≤ 0.05 by ANOVA followed by Duncan's multiple range test



arsenic-treated mutant lines but only at 20 DAC. Increasing SOD activity continuously generated H<sub>2</sub>O<sub>2</sub>, which could not be counterbalanced by abrupt decrease in APX and CAT. Declining level of APX might be due to nearly twofold decrease in AsA level and its redox state in B1 and BioL-212. Besides serving as co-factor of APX, AsA itself

can detoxify ROS through non-enzymatic mechanism [38, 44] and its availability and redox state plays key roles in signaling network through controlled metabolism of H<sub>2</sub>O<sub>2</sub> during metal stress [6]. H<sub>2</sub>O<sub>2</sub> has a longer half-life than superoxides [4, 14, 20], and therefore, its initial surge in leaves of arsenic-treated mutants indicated early “oxidative

**Table 2** Effect of arsenic (As) treatments (30 mg l<sup>-1</sup>) on reduced (AsA), redox state of ascorbate and activities of superoxide dismutase (SOD, U min<sup>-1</sup> mg<sup>-1</sup> protein), ascorbate peroxidase (APX, nmol AsA oxidized min<sup>-1</sup> mg<sup>-1</sup> protein) and catalases (CAT, nmol H<sub>2</sub>O<sub>2</sub>

degraded min<sup>-1</sup> mg<sup>-1</sup> protein) in leaves of control and four treated genotypes at control (0) and 20, 40 and 60 days after commencement of treatment (DAC) of *L. sativus* L.

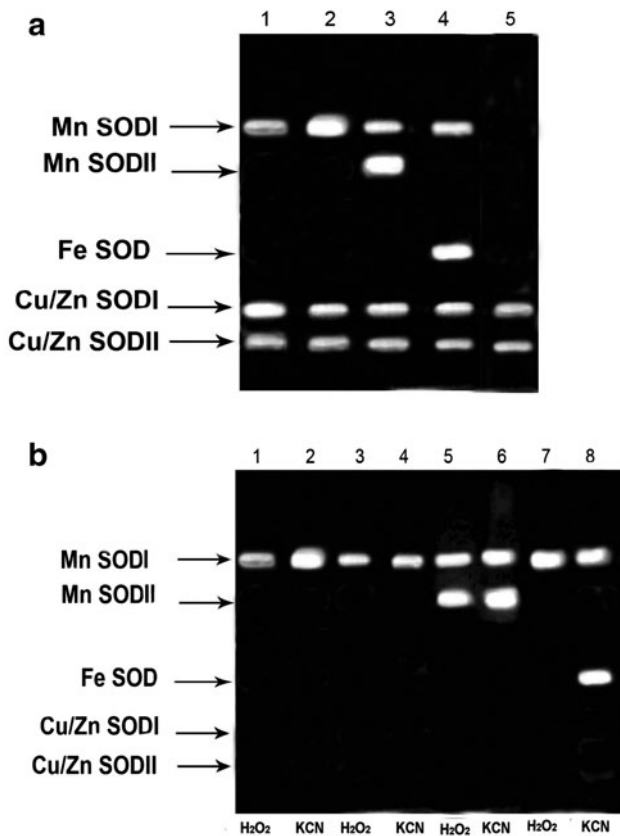
Parameters	DAC	B1	BioL-212	BSM	PIM
AsA (μmol g <sup>-1</sup> FW)	0	1.81 ± 10.4ab'	1.75 ± 10.6bb'	1.89 ± 12.4aa'	1.69 ± 11.7ba'
	20	3.39 ± 14.8aa'	2.98 ± 10.3aa'	1.18 ± 17.3bb'	1.07 ± 7.8bb'
	40	1.10 ± 7.3cc'	3.12 ± 13.0aa'	1.76 ± 8.8ba'	1.65 ± 7.2ba'
	60	0.91 ± 7.9cd'	0.98 ± 10.0cc'	1.83 ± 7.9aa'	1.71 ± 8.1ba'
AsA redox [AsA/(AsA + DHA)]	0	0.856 ± 0.09a a'	0.891 ± 0.09aa'	0.910 ± 0.08aa'	0.913 ± 0.10aa'
	20	0.932 ± 0.11aa'	0.943 ± 0.10aa'	0.657 ± 0.12bb'	0.639 ± 0.09bb'
	40	0.542 ± 0.08bb'	0.951 ± 0.09aa'	0.868 ± 0.09aa'	0.890 ± 0.11aa'
	60	0.461 ± 0.09bc'	0.458 ± 0.11bb'	0.905 ± 0.09aa'	0.915 ± 0.12aa'
SOD	0	111.5 ± 5.1ac'	118.9 ± 4.9ac'	113.3 ± 4.7ab'	111.5 ± 5.5a b'
	20	110.9 ± 5.8cc'	169.8 ± 4.8bb'	189.7 ± 5.1aa'	191.6 ± 7.7aa'
	40	148.8 ± 4.8bb'	191.8 ± 10.5aa'	193.6 ± 9.8aa'	189.8 ± 8.5aa'
	60	205.6 ± 11.6aa'	190.6 ± 9.8aa'	195.1 ± 6.9aa'	197.5 ± 10.2aa'
APX	0	175.8 ± 7.8ba'	169.9 ± 8.1bb'	180.5 ± 8.9ac'	181.7 ± 10.0ac'
	20	174.7 ± 8.6ba'	337.7 ± 12.6aa'	60.3 ± 4.3cd'	51.73 ± 4.5dd'
	40	77.9 ± 4.9cb'	329.6 ± 9.3ba'	450.4 ± 18.5aa'	458.8 ± 13.2aa'
	60	58.7 ± 6.1cc'	109.3 ± 5.5bc'	324.5 ± 11.3ab'	327.1 ± 10.9ab'
CAT	0	39.1 ± 4.7aa'	40.1 ± 6.7aa'	37.7 ± 5.1ab'	39.8 ± 5.6ab'
	20	40.5 ± 3.8aa'	38.8 ± 6.4aa'	21.6 ± 4.9bc'	19.9 ± 5.7bc'
	40	40.1 ± 4.1ba'	40.3 ± 4.7ba'	51.5 ± 5.3aa'	53.7 ± 6.0aa'
	60	36.7 ± 5.0ba'	18.9 ± 5.1cb'	58.6 ± 6.1aa'	57.7 ± 5.9aa'

Data were presented as mean ± SE of four replicates. Different small letters in each row indicate significant differences among genotypes, and letters with prime in each column indicate significant differences among treatments (for a particular trait) at  $P \leq 0.05$  by ANOVA followed by Duncan's multiple range test

burst" in the photosynthetic organs, which might trigger enhanced defense response against excess ROS at the later stages of growth. Rise in APX activity powered by high AsA redox and increasing CAT level at 40 DAC and at 60 DAC was the strong evidence of this enhanced response. This might be instrumental to maintain H<sub>2</sub>O<sub>2</sub> in mutants at a particular level, which is still significantly higher than control but was considerably lower than that of both varieties and was not toxic to normal reproductive growth of treated mutants, as was also observed in cadmium-treated lentil seedlings [45] and during arsenic-induced induction of *Fusarium* wilting in grass pea genotypes [48]. Certainly, the status of cellular H<sub>2</sub>O<sub>2</sub> informed the plant cell about the severity of the oxidative stress and hence the appropriate level of antioxidant enzyme activities through induction [20, 32, 48]. Present results indicate criticality of H<sub>2</sub>O<sub>2</sub> level in stress perception, which was regulated/adjusted by tolerant genotypes in favor of their growth and yield performances during prolonged exposure to arsenic. This was strongly suggested by the absence of oxidative stress symptom (normal MDA and EL%) in shoots of both BSM and PIM lines despite the high H<sub>2</sub>O<sub>2</sub> content at 60 DAC. Huge enhancement in MDA content and EL% along with

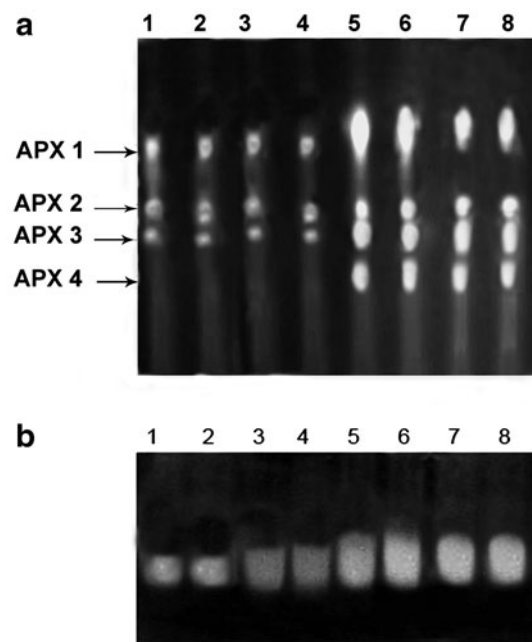
extremely high H<sub>2</sub>O<sub>2</sub> content in leaves of both the varieties at this stage resulted in onset of arsenic-induced oxidative stress, which jeopardized growth and yield potential of both varieties, similar to reports in different other crop plants under arsenic exposure [12, 15, 17, 18, 34–37, 46, 47].

Changes in antioxidant enzyme activities coincided with a variable increase or decrease in their individual isoform expressions. The intensity of enzyme bands color can reflect relative quantity of the isozyme activity. Analysis of isoform and inhibitor studies revealed that the significant enhancement in SOD activity was mainly due to appearance of Mn-SOD I isoform in B1 and BioL-212, Mn-SOD I and II isoforms in BSM and Mn-SOD I, and one Fe-SOD isoforms in PIM line. The supply of arsenic reportedly enhanced the activity of Cu/Zn-SOD II in *Trifolium pratense*, although no new isoforms was detected [28]. In *Arabidopsis*, transcripts for genes encoding a chloroplastic and a cytosolic Cu/Zn-SOD were induced by As (V) exposure, while transcripts for a Fe-SOD were down-regulated [1]. Origin of three novel isoforms in the present study indicated the involvement of SOD expressions in different cellular compartments to combat the elevated



**Fig. 2** **a** Activity gel of superoxide dismutase (SOD) in native PAGE on 10 % acrylamide gels of leaf extracts of four grass pea genotypes at 60 days after commencement of arsenic (sodium arsenate 30 mg As I<sup>-1</sup>) treatment; *lane 1* variety B1, *lane 2* variety BioL-212, *lane 3* bold-seeded mutant, *lane 4* pod indehiscent mutant and *lane 5* control plants, and **b** inhibitor studies with H<sub>2</sub>O<sub>2</sub> and KCN and visualization of SOD isoforms in native PAGE of leaf extracts of grass pea (*L. sativus* L.) variety B1 (*lanes 1* and 2), variety BioL-212 (*lanes 3* and 4), bold-seeded mutant (*lanes 5* and 6) and pod indehiscent mutant (*lanes 7* and 8) at 60 days after commencement of treatment. The control plants containing only Cu/Zn-SOD I and II showed no bands in inhibitor study, and thus not presented

generation of superoxide radicals due to arsenic exposure and suggested that grass pea lines are quite able to protect their important organelles, such as mitochondria and peroxisomes, against the detrimental effects of ROS. Interestingly enough, a recent study demonstrated that arsenic exposure significantly upregulated Cu/Zn-SODs, Fe-SODs and Mn-SODs activities in *Phaseolus vulgaris* genotype VL-63 but strictly regulated SOD activity in its two arsenic-tolerant SOD-deficient mutant lines by upregulating Cu/Zn-SODs expression on the one hand and down-regulating Mn-SOD and Fe-SOD on the other hand [49]. For APX, the changes in activity were mainly due to enhanced/reduced expression of APX 1, APX 2 and APX 3 isozymes. The visualization of APX 4 as a unique isoform in BSM and PIM lines indicated intrinsic modulation of major H<sub>2</sub>O<sub>2</sub>-scavenging enzyme in the photosynthetic



**Fig. 3** Effects of As treatments (sodium arsenate, 30 mg As I<sup>-1</sup>) on isozymes banding of **a** APX activity in native PAGE on 4 % acrylamide gels of leaf extracts of grass pea genotypes at 60 days after commencement of treatment; *lane 1* control plant, *lane 2* variety B1, *lanes 3* and 4 variety BioL-212, *lanes 5* and 6 bold-seeded mutant and *lanes 7* and 8 pod indehiscent mutant, and **b** CAT activity in native PAGE on 6 % acrylamide gels of leaf extracts of grass pea genotypes at 60 days after commencement of treatment; *lane 1* control plants, *lane 2* variety B1, *lanes 3* and 4 variety BioL-212, *lanes 5* and 6 bold-seeded mutant, and *lanes 7* and 8 pod indehiscent mutant

organ of the mutant plants. Upregulation of SODs and APX isoforms was also reported in rice seedlings, which was more pronounced in As (III) treatment compared to As (V) [37]. By contrast, transgenic tall fescue plants expressing the Cu/Zn-SOD and APX genes in chloroplasts exhibited downregulation of enzyme isoforms upon submission to arsenic treatment [23]. In wheat seedlings, arsenic induced the expression of CAT isozymes but inhibited the expression of SOD and APX isozymes at concentrations lower than 1 mg kg<sup>-1</sup>, while opposite result was obtained at high concentrations [24]. In the present study, increasing staining intensity confirmed the enhanced level of CAT expression in the treated mutants, while decreasing activity was manifested by declining band intensity in BioL-212 after prolonged arsenic exposure. Obviously, increased isozyme activity and/or appearance of novel isoforms in APX and CAT conferred both the mutants' appreciable capability of H<sub>2</sub>O<sub>2</sub> scavenging to counterbalance enhanced SOD-generated H<sub>2</sub>O<sub>2</sub> production during arsenic treatment. The variations in the isozyme pattern and their correspondence to total assayed activity suggested that the three H<sub>2</sub>O<sub>2</sub>-metabolizing enzymes



responded strongly to arsenic-induced oxidative stress in grass pea genotypes.

## Conclusion

Results revealed significant genotypic differences in response to 30 mg As I<sup>-1</sup> treatment and even differences between growth stages. Arsenic treatment inhibited growth and yield potential of variety B1 and BioL-212, while no such effect was observed in BSM and PIM lines. Present results also revealed the importance of reproductive stage in maintaining normal growth, photosynthesis and seed yield during prolonged arsenic exposure by mitigating oxidative damage through the modulation of H<sub>2</sub>O<sub>2</sub> metabolism in fine tune. Isozyme analysis revealed origin of unique isoforms of SOD and APX in response to arsenic treatment and increase in activity of existing isoforms of SOD, APX and CAT, conferring enhanced activity.

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