Modulation of antioxidant enzymes by salicylic acid in arsenic exposed *Glycine max* L.

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Abstract

To investigate the physiological and metabolic attributes of arsenic (As) stress tolerance conferred by exogenous salicylic acid (SA), *Glycine max* L. (variety JS 335) seeds were aseptically germinated over filter paper moistened with SA (500 μ M) and/or 10 and 100 μ M As (Sodium arsenite was used). On 2nd and 5th days of germination, the growing radicles were harvested, and analyzed for growth and different metabolic attributes. Findings exemplified that As significantly decreased germination percentage, radicle length, dry mass and activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX), while stimulated the contents of As, reactive oxygen species (ROS), lipoxygenase (LOX), guaiacol peroxidase (POD) and proline. Additionally, isozymes of antioxidants were also scrutinized over Native-PAGE gels and were found to be altered considerably under As-stress. However, exogenous SA remarkably enhanced germination percentage, growth indices, activities of SOD, CAT and APX, and proline accumulation along with reduced As, ROS and LOX, and restoring POD in As-stressed seedlings. In conclusion, SA confers As-stress tolerance to *Glycine max* L. by regulating the antioxidant enzymes and proline accumulation thereby reduced As content and ROS production. Further study is intended, particularly at gene level, to understand precise mechanism(s) involved in SA-mediated As-stress tolerance.

Keywords: Antioxidant enzymes, arsenic toxicity, oxidative stress, reactive oxygen species, salicylic acid

1. Introduction

Arsenic (As) is one of the non-essential metalloid present ubiquitously in nature. It is highly toxic to both animals and plants and has no known beneficial biological function (Armendariz *et al.*, 2016). Both natural and anthropogenic activities, such as, mining, semi-conductor manufacturing, irrigation by As contaminated water, use of fossil fuels and As based pesticides/ fertilizers in agriculture, and waste disposal lead to severe contamination by it in the surrounding environment (Chandrakar *et al.*, 2016). Arsenic exists in both organic and inorganic forms, out of which the later is predominantly present in the environment, particularly as arsenite (As^{III}) and arsenate (As^{V}) (Armendariz *et al.*, 2016). Arsenate is a chemical analogue of phosphate and is taken up by plant roots through high affinity phosphate uptake systems, whereas, As^{III} is taken up *via* aquaglyceroporins (Chandrakar *et al.*, 2016). Between the two, As^{III} is 100-fold more toxic than As^{V} , due to its lovingness towards -SH groups of both enzymes and proteins leading to inhibition of cellular functions, which ultimately results in cell death. Plants, on exposure to phytotoxic amounts of As, induce overproduction of reactive oxygen species (ROS), such as, superoxide anion ($\text{O}_2^{\cdot-}$), hydroxyl radical ($\text{OH}^{\cdot}$) and hydrogen peroxide (H_2O_2), resulting in oxidative stress imposing array of irreparable injuries (Kaur *et al.*, 2012). These ROS are largely shown to react with all sorts of cellular macromolecules like lipids, carbohydrates, proteins and nucleic acids (Parkhey *et al.*, 2014a). Moreover, oxidation of lipid moiety can also be initiated enzymatically by lipoxygenase (LOX) and is believed to be an important factor of growth inhibition in plants exposed to heavy metals (Mostofa and Fujita, 2013). In order to combat against the excessive ROS and to protect the cells under oxidative environments, plant cells possess a complex network of defense system, which comprises both

enzymatic and non-enzymatic components (Chandra and Keshavkant, 2016; Chandrakar *et al.*, 2016). The enzymatic component includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (POD) and ascorbate peroxidase (APX) (Dong *et al.*, 2014). This function may also be achieved by a concerted action of low molecular weight non-enzymatic antioxidants, such as, α -tocopherol, proline, ascorbate, glutathione and phenolic compounds (Raza *et al.*, 2014; Singh *et al.*, 2015b). The very first step taken towards detoxification of cellular ROS is by SOD (Keshavkant and Naithani, 2001). SOD is one of the metalloenzymes that leads conversion of two $\text{O}_2^{\cdot-}$ radicals into H_2O_2 and O_2 (Keshavkant and Naithani, 2001). CAT and POD catalyzes the breakdown of H_2O_2 (Chandra and Keshavkant, 2016). Although, CAT is apparently absent in the chloroplasts, H_2O_2 may be detoxified in a reaction catalyzed by an ascorbate-specific peroxidase that is normally found in abundance in this organelle through the ascorbate-glutathione cycle (Chandrakar *et al.*, 2016).

Salicylic acid (SA) has been considerably recognized as an endogenous natural signal molecule involved in plant's defense mechanisms by regulating both physiological and biochemical processes (Odjegba, 2012; Dong *et al.*, 2015). Further, it is also well documented that SA enhances accumulation of proline thereby minimizes toxic effects of metals/metalloids (Mostofa and Fujita, 2013). All these researches demonstrated that SA-regulated abiotic stress tolerance in plants are involved in antioxidant responses, thus suggesting that protection of plants from oxidative damage by SA is intimately linked with an enhanced antioxidant system (Parkhey *et al.*, 2014b). A recent report demonstrated that co-application of SA with As, was more effective in reducing metalloid exerted oxidative injury than its pre-treatment (Singh *et al.*, 2015a).

The present research was aimed to 1) analyze the physiological effects of As in *Glycine max* L. seeds/seedlings, 2) investigate the levels of As content, $O_2^{\cdot-}$, $OH^{\cdot}$ and H_2O_2 , 3) determine changes in the activities of various enzymes (SOD, CAT, POD, APX and LOX), 4) isozyme profiling of antioxidant enzymes, 5) monitor change in proline content, and 6) modulation of As-induced stress responses by exogenous addition of SA.

2. Materials and Methods

2.1. Plant material, treatments and growth analysis

Glycine max L. (variety JS 335) seeds were washed initially with 1% (v/v) sodium hypochlorite solution for 5 min following washing (5 times) with MilliQ water (MW) (Millipore, Gradient A-10, USA). Primarily, disinfected seeds were germinated over two layers of filter paper towels, pre-soaked with a series of As (Sodium arsenite, used as a source of As) solution *s.i.e.*, 10, 25, 50, 75, 100 and 125 μ M, in germination boxes of 26 x 16 x 3 cm size (Parkhey *et al.*, 2014a). These boxes were kept in darkness at room temperature (26–28 °C). Respective growth medium (10 mL) was supplied to the germinating seeds in each 30 h. Looking to the germination count, two concentrations *i.e.* 10 and 100 μ M As, were then selected for current study, where seeds revealed 00% (but the rate of germination was slow compared to MW-grown control seeds) and 74% reductions in germination percentage, respectively on 5th day of investigation. Further, a dose of SA fixed was 500 μ M, based on screening experiments. In a preliminary investigation, adding various concentrations of SA (100, 200, 300 and 400 μ M) partially rescued the adverse impacts of As on growth in *Glycine max* L., but 500 μ M SA was found to confer maximum tolerance (as evidenced by 45% rise in biomass) towards As (100 μ M) toxicity. Hence, 500 μ M SA was used in this entire experiment.

A randomized block design comprising of a control (C, without As and SA) and five treatments; 500 μ M SA (T1), 10 μ M As (T2), 10 μ M As + 500 μ M SA (T3), 100 μ M As (T4), and 100 μ M As + 500 μ M SA (T5) were used. Sixty sterilized seeds were then placed for germination in each of the treatments, following the procedure of Parkhey *et al.* (2014a), as mentioned previously. On 2nd and 5th days of sample incubation, germination percentage was assessed and then their radicles were removed carefully. The lengths of the ten randomly selected radicles were measured with the help of a scale (mm), to record smallest change in their lengths. Likewise, ten radicles were pooled in each replicate and weighed in an electronic balance (Sartorius, Sweden). Dry mass (DM) of these radicles was measured after placing them in a hot air oven at 60 °C for 72 h. Each experiment was performed in five replicates. Left over radicles were stored in sterile vials at -80 °C (U410, Eppendorf, Germany) for further analyses.

All the chemicals used in this study are of standard purity and analytical grades, supplied by Qualigens and Merck, India, or Sigma, USA.

2.2. Arsenic content

To determine As content, weighed amounts (0.1 g) of dried radicles were digested using HNO_3 : H_2O_2 : H_2O in the ratio of 5:1:1 (v:v:v) at 80 °C until a transparent solution was obtained (Lozano-Rodriguez *et al.*, 1995). The volume of digested sample was made up to 15 mL with MW, and amount of As in it was monitored using atomic absorption spectrometer coupled to a hydride generation system (Agilent, AA240, USA). The standard reference materials of metals (Merck, Darmstadt, Germany) were used for calibration and quality assurance for analysis.

2.3. Reactive oxygen species

$O_2^{\cdot-}$ content was measured following the method of Schopfer *et al.* (2001). Excised radicles (0.2 g) were imbibed in 20 mM phosphate buffer, pH 6, consisting 2, 3-Bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (500 μ M) in dark at 26 °C, on a shaker at 50 rpm. Absorbance of the soaking medium was read at 470 nm in UV-Vis spectrophotometer (Lambda 25, Perkin Elmer, USA). $O_2^{\cdot-}$ content was calculated using an extinction coefficient $2.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ and referred as $\mu\text{mol min}^{-1} \text{ g}^{-1}$ fresh mass (FM).

To measure $OH^{\cdot}$, radicles (0.2 g) were homogenized in 2 mL of phosphate buffer (10 mM, pH 7.4) containing 15 mM 2-deoxyribose and incubated at 37 °C for 2 h. An aliquot of above solution was mixed with 3 mL thiobarbituric acid (0.5% (w/v) in 5 mM NaOH) and 1 mL glacial acetic acid (Kaur *et al.*, 2012). Now, this solution was heated at 100 °C for 30 min and then cooled at 4 °C for 10 min. Absorbance was recorded at 532 nm and corrected for non-specific absorbance at 600 nm. Content of $OH^{\cdot}$ was calculated using an extinction coefficient of $0.155 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as nmol g^{-1} FM.

To assess H_2O_2 content, weighed amounts (0.2 g) of radicles were extracted with 2 mL of 0.1% (w/v) trichloroacetic acid (TCA) and centrifuged at 12,000 rpm for 15 min at 25 °C (Velikova *et al.*, 2000). An aliquot of supernatant was added to equal volumes of 10 mM phosphate buffer (pH 7) and potassium iodide (1 M). Now, absorbance was read at 390 nm. H_2O_2 content was calculated using an extinction coefficient $0.28 \mu\text{M}^{-1} \text{ cm}^{-1}$ and was expressed as $\mu\text{mol g}^{-1}$ FM.

2.4. Determination of LOX activity

LOX (EC 1.13.11.12) was assayed according to the method of Olaiya (2010). For this, the substrate was prepared by adding 10 μ L linoleic acid to 25 mL sodium tetraborate (0.1 M) containing 0.1% (v/v)

Tween-20. Activity was determined by adding 100 μ L each of enzyme extract and substrate to 2.9 mL phosphate buffer (0.1 M, pH 4.5). The absorbance was recorded at 234 nm. LOX activity was calculated using the extinction coefficient $0.025 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as $\mu\text{mol min}^{-1} \text{ mg}^{-1}$ protein.

2.5. Assay of antioxidant enzymes

Samples (0.2 g) were extracted with 2 mL of 10 mM phosphate buffer (pH 7.2), containing 1 mM EDTA, 2 mM DTT and 0.2% (v/v) Triton X-100 and centrifuged at 14,000 rpm for 20 min at 4 °C. Supernatant thus obtained was used for estimations of protein and antioxidants.

Protein content was assessed following the procedure of Bradford (1976). Bovine serum albumin was used to prepare standard curve.

Following the method of Marklund and Marklund (1974), activity of SOD (EC 1.15.1.1) was determined by estimating the percent inhibition of pyrogallol auto-oxidation by the enzyme at 420 nm. For this, 2.74 mL of Tris-HCl buffer (50 mM, pH 8.2) containing 1mM each of DETAPAC and EDTA was taken in a test tube, to which 0.2 mL of enzyme extract was added. The reaction was initiated by adding 60 μ L of pyrogallol (0.2 mM of pyrogallol dissolved in 10 mM HCl) and the change in absorbance was recorded at 420 nm after 6 min of incubation at room temperature. The activity of enzyme was expressed as Units of SOD $\text{min}^{-1} \text{ mg}^{-1}$ protein.

Activity of CAT (EC 1.11.1.6) was assayed following the method of Chance and Maehly (1955). For its estimation, the decomposition of H_2O_2 was measured by recording the fall in absorbance at 240 nm. Assay mixture comprised of 2.74 mL of potassium phosphate buffer (37.5 mM, pH 6.8) and 60 μ L of extract. Enzyme activity was triggered by adding 200 μ L of H_2O_2 (60 mM). The change in absorbance was record-

ed for 5 min at an interval of 15 sec. Activity of CAT was calculated using an extinction coefficient of $0.039 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as $\text{nmol min}^{-1} \text{ mg}^{-1}$ protein.

POD (EC 1.11.1.7) activity was measured following the method of Chance and Maehly (1955). In a test tube, 2.3 mL of phosphate buffer (0.02 M, pH 6.4), 0.5 mL guaiacol (12 mM) and 0.2 mL enzyme extract were taken. Reaction was triggered by adding 20 μL of 3% (v/v) H_2O_2 . The change in absorbance was read at 470 nm after 3 min of incubation at room temperature, and activity was expressed in terms of $\text{mmol min}^{-1} \text{ mg}^{-1}$ protein.

Activity of APX (EC 1.11.1.11) was assayed according to Nakano and Asada (1981) by monitoring the rate of ascorbate oxidation at 290 nm. The reaction mixture comprised of 2.3 mL of potassium phosphate buffer (0.025 M, pH 7.0), 500 μL of ascorbic acid (0.0025 M), 190 μL of EDTA (0.001 M) and 10 μL of isolated enzyme. Immediately after the addition of 10 μL of H_2O_2 (0.1 M) into assay mixture, initial absorbance was measured at 290 nm. After 20 min of incubation at room temperature, absorbance of the mixture was once again recorded. Enzyme activity was calculated following the extinction coefficient of $0.0028 \text{ M}^{-1} \text{ cm}^{-1}$ and expressed as $\text{mmol min}^{-1} \text{ mg}^{-1}$ protein.

2.6. Isozyme analysis

Electrophoretic separation of SOD, CAT, POD and APX were performed on native polyacrylamide gels (10%) using Tris-glycine buffer (5 mM, pH 8.3) (in case of APX, running buffer consists of 4 mM ascorbate), at 4 °C for 2 h with a constant current of 20 mA, using Mini-Protean tetra cell (BioRad, USA). After complete run, gels were imaged and analyzed using a Gel-Doc system (BioRad, USA). To visualize the SOD, gels were incubated in the dark for 20 min in the 2.45 mM nitro blue tetrazolium (NBT) solution, and were then immersed in 36 mM dipotassium hydrogen phosphate (pH 7.8) containing 28 μM ribofla-

vin and 28 mM TEMED, until the gel turns blue except the region showing SOD activity (Chun-xi *et al.*, 2007). Isozymes of CAT were stained following the method described by Woodbury *et al.* (1971). The gels were incubated in 0.03% (v/v) H_2O_2 solution for 10 min. The gels were rinsed quickly in MW and stained in a solution containing 1% (w/v) each of potassium ferricyanide and ferric chloride. As soon as a green colour began to appear, gels were washed with MW. Isozymes of POD were revealed following Srivastava and Huystee (1977). The gels were equilibrated with 100 mM potassium phosphate buffer (pH 6.5) for 15 min and then in 12.5 mM guaiacol solution containing benzidine (1.7 mM) and H_2O_2 (12 mM). After gentle shaking, brown coloured bands appeared against a clear background. For APX detection, gels were equilibrated with 50 mM sodium phosphate buffer (pH 7) and 2 mM ascorbate for 30 min (Mittler and Zilinskas, 1993). Afterwards, the gels were incubated with 50 mM sodium phosphate buffer (pH 7) containing 4 mM ascorbate and 4 mM H_2O_2 for 20 min. Finally, gels were washed twice with sodium phosphate buffer (50 mM, pH 7) and stained in 50 mM sodium phosphate buffer (pH 7.8) containing 28 mM TEMED and 2.45 mM NBT. The reaction was continued for 10-15 min and stopped by a brief wash with MW.

2.7. Proline determination

Proline was estimated according to Bates *et al.* (1973). 0.5 g radicle was homogenized with 10 mL of 3% (w/v) sulfo-salicylic acid and centrifuged at 6,000 rpm for 15 min at 26 °C. Supernatant (2 mL) was mixed with 2 mL each of ninhydrin reagent and glacial acetic acid. The mixture was incubated at 100 °C for 60 min, and cooled at room temperature. Then, 4 mL toluene was added and the chromophore containing toluene was aspirated out and its absorbance was recorded at 520 nm taking toluene as a blank. Its amount was expressed as mg g^{-1} FM.

2.8. Statistical analysis

All the investigations were performed twice with five separate replications. The data obtained were subjected to one-way ANOVA, and the mean differences were compared by Duncan's multiple range tests using SPSS software (Ver. 16.0).

3. Results

3.1. Growth analysis

Growth indices of *Glycine max* L. radicles were negatively influenced by As treatment. The 10 μM (T2) As slightly reduced the germination rate (8%), length (40%), and DM (25%) of the radicles, as compared to the MW-treated control, on 2nd day of investigation (Table 1). These reductions were found to be substantially high (germination: 74%, radicle length: 88% and DM: 83%) under T4 (100 μM As) treatment, on 5th day of analysis, compared to the control (Table 1).

On the other hand, SA (T1), as seed soaking in absence of As, stimulated the growth traits ($P < 0.05$) higher than the controls. SA, also uplifted the growth of the seedlings grown in T3 (10 μM As + 500 μM SA) and T5 (100 μM As + 500 μM SA) and the data obtained were statistically significant ($P < 0.05$) than those of the seeds grown under As alone (T2 and T4) (Table 1).

3.2. Arsenic content

Considerable ($P < 0.05$) rise in As accumulation was measured in respect to both concentration and length of exposure (Table 1). Amounts of As measured in the radicles of T2 (10 μM As) and T4 (100 μM As) were 20.41 $\mu\text{g As g}^{-1}$ DM and 23.54 $\mu\text{g As g}^{-1}$ DM respectively, on 2nd day, while with increased exposure, they were 24.89 $\mu\text{g As g}^{-1}$ DM and 31.97 $\mu\text{g As g}^{-1}$ DM respectively, on 5th day of investigation. However, an exogenous addition of SA caused limited (7-27%) accumulation of As in T2 and T4 treated *Glycine max* L. (Table 1).

Table 1. Effects of arsenic and/ or salicylic acid treatments on germination percentage, radicle length, dry mass, arsenic and proline content of *Glycine max* L. on 2nd and 5th days of exposure.

Treatments	Germination (%)		Radicle length (mm)		Dry mass (g)		As content ($\mu\text{g g}^{-1}$ DM)		Proline (mg g^{-1} FM)	
	2 nd Day	5 th Day	2 nd Day	5 th Day	2 nd Day	5 th Day	2 nd Day	5 th Day	2 nd Day	5 th Day
C	90 ^b $\pm$ 0.57	100 ^a $\pm$ 0	15 ^b $\pm$ 1	75 ^c $\pm$ 2	0.04 ^b $\pm$ 0.005	0.12 ^b $\pm$ 0.005	-	-	0.2 ^f $\pm$ 0.01	0.31 ^e $\pm$ 0.006
T1	100 ^a $\pm$ 0	100 ^a $\pm$ 0	18 ^a $\pm$ 2	86 ^a $\pm$ 1	0.06 ^a $\pm$ 0.005	0.13 ^a $\pm$ 0.01	-	-	0.23 ^e $\pm$ 0.002	0.36 ^e $\pm$ 0.02
T2	83 ^c $\pm$ 1	100 ^a $\pm$ 0	9 ^c $\pm$ 1	41 ^d $\pm$ 1	0.03 ^c $\pm$ 0.005	0.08 ^c $\pm$ 0.005	20.41 ^b $\pm$ 0.05	24.89 ^b $\pm$ 0.05	0.31 ^d $\pm$ 0.04	0.47 ^d $\pm$ 0.05
T3	90 ^b $\pm$ 2	100 ^a $\pm$ 0	16 ^{ab} $\pm$ 1	80 ^b $\pm$ 2	0.04 ^b $\pm$ 0.005	0.12 ^b $\pm$ 0.01	14.79 ^c $\pm$ 0.05	18.43 ^c $\pm$ 0.01	0.53 ^c $\pm$ 0.07	0.93 ^{bc} $\pm$ 0.005
T4	13 ^e $\pm$ 3	26 ^c $\pm$ 3	2 ^e $\pm$ 1	9 ^f $\pm$ 3	0.006 ^e $\pm$ 0.005	0.02 ^d $\pm$ 0.01	23.54 ^a $\pm$ 0.03	31.97 ^a $\pm$ 0.06	1.12 ^b $\pm$ 0.01	2.05 ^b $\pm$ 0.19
T5	35 ^d $\pm$ 5	70 ^b $\pm$ 2	8 ^d $\pm$ 1	34 ^e $\pm$ 4	0.01 ^d $\pm$ 0.005	0.03 ^d $\pm$ 0.005	21.77 ^b $\pm$ 0.05	25.93 ^b $\pm$ 0.05	1.49 ^a $\pm$ 0.03	2.66 ^a $\pm$ 0.03

In this table, C = control, T1 = 500 μM SA, T2 = 10 μM As, T3 = 10 μM As + 500 μM SA, T4 = 100 μM As and T5 = 100 μM As + 500 μM SA. Data are mean $\pm$ SD of five individual replicates. Means followed by small letters are significantly different at $P < 0.05$

Table 2. Amounts of superoxide anions, hydroxyl radicals, hydrogen peroxide and lipoxygenase in *Glycine max* L. radicles, harvested after 2nd and 5th days of growth under experimental solutions.

Treatments	Superoxide ($\mu\text{mol min}^{-1} \text{g}^{-1} \text{FM}$)		Hydroxyl radical ($\text{nmol g}^{-1} \text{FM}$)		Hydrogen peroxide ($\mu\text{mol g}^{-1} \text{FM}$)		Lipoxygenase ($\mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$)	
	2 nd Day	5 th Day	2 nd Day	5 th Day	2 nd Day	5 th Day	2 nd Day	5 th Day
C	0.84 ^a ± 0.18	1.68 ^a ± 0.79	5.47 ^a ± 1.39	8.46 ^a ± 0.07	0.23 ^a ± 0.009	0.36 ^a ± 0.007	0.46 ^a ± 0.03	0.61 ^a ± 0.16
T1	0.51 ^a ± 0.13	1.39 ^a ± 0.16	3.33 ^a ± 0.36	6.03 ^a ± 0.09	0.07 ^a ± 0.004	0.2 ^a ± 0.009	0.27 ^a ± 0.11	0.40 ^a ± 0.08
T2	2.52 ^c ± 0.31	8.87 ^c ± 1.27	8.96 ^c ± 0.42	10.86 ^c ± 0.18	0.42 ^c ± 0.008	0.54 ^c ± 0.004	1.56 ^c ± 0.09	1.83 ^c ± 0.16
T3	1.96 ^b ± 0.54	6.73 ^b ± 0.25	6.4 ^b ± 0.08	8.71 ^b ± 0.21	0.26 ^b ± 0.007	0.38 ^b ± 0.005	0.77 ^b ± 0.03	1.18 ^b ± 0.03
T4	20.62 ^a ± 1.42	24.2 ^a ± 0.48	23.96 ^a ± 0.36	28.71 ^a ± 0.05	0.79 ^a ± 0.007	1.24 ^a ± 0.008	4.46 ^a ± 0.15	5.15 ^a ± 0.65
T5	17.42 ^b ± 1.25	21.84 ^b ± 0.8	17.24 ^b ± 0.18	21.5 ^b ± 0.48	0.63 ^b ± 0.006	1.08 ^b ± 0.008	3.39 ^b ± 0.45	4.16 ^b ± 0.22

In this table, C = control, T1 = 500 μM SA, T2 = 10 μM As, T3 = 10 μM As + 500 μM SA, T4 = 100 μM As and T5 = 100 μM As + 500 μM SA. Data presented are mean $\pm$ SD of five separate observations. Different letters indicate significant difference between treatments at $P < 0.05$

3.3. Reactive oxygen species

Increases in As concentration from 10 μM (T2) to 100 μM (T4), significantly ($P < 0.05$) enhanced the levels of all the three ROS ($\text{O}_2^{\cdot-}$: 801-2880%, $\text{OH}^{\cdot}$: 198-524%, H_2O_2 : 234-539%), compared to the non-treated controls, on 5th day of analysis (Table 2). Data also indicated that As-induced damage in *Glycine max* L. is mitigated by exogenous SA up to a large extent ($P < 0.05$) by controlling As uptake (7-27%), thereby reducing the ROS accumulation (Table 2). Least levels of $\text{O}_2^{\cdot-}$ (0.51 $\mu\text{mol min}^{-1} \text{g}^{-1} \text{FM}$), $\text{OH}^{\cdot}$ (3.33 $\text{nmol g}^{-1} \text{FM}$) and H_2O_2 (0.07 $\mu\text{mol g}^{-1} \text{FM}$) were recorded in SA (T1) grown seedlings (Table 2).

3.4. Lipoxygenase

Alike ROS, T2 (10 μM As) and T4 (100 μM As) significantly (503-1193%, $P < 0.05$) stimulated the LOX also, on 2nd day of investigation, and was found to be increased further with extended exposure ($r = 0.97$, $P < 0.05$) and As accumulation ($r = 0.81$, $P < 0.05$) (Table 2). However, supplementation of SA along with As (T3 and T5) declined (almost up to 50%) the LOX activity in *Glycine max* L. (Table 2).

3.5. Antioxidants

Activities of SOD, CAT, POD and APX in response to As (T2: 10 μM As, and T4: 100 μM As) and its combination with SA (T3: 10 μM As + 500 μM SA and T5: 100 μM As + 500 μM SA) are observed to perform differently (Figures 1, 3, 5 and 7). Activities of SOD, CAT and APX were decreased (73%, 75% and 28%, respectively) significantly ($P < 0.05$), while POD increased (40%) with advancing (24.89 $\mu\text{g As g}^{-1} \text{DM}$ to 31.97 $\mu\text{g As g}^{-1} \text{DM}$) As accumulation. However, addition of SA either stimulated their activities over the values of the non-treated control or restored over those values recorded in As alone (T2 and T4) treated seedlings.

In gel activity analysis of SOD unveiled a total of seven isoforms in response to As and/or SA. Exposure to T2 (10 μM As) and T4 (100 μM As) led decline in the intensities of isozymes-I to IV on 5th day of investigation, compared to 2nd day (Figure 2). Additionally, intensities of isoforms-I and III were reduced in T2 and T4 as compare to MW-treated control on 2nd day of treatment. In contrast, isozyme-I displayed increased activity in response to SA, and was also supported by biochemical data (Figures 1 and 2).

Figure 1. Change in the activity of superoxide dismutase in *Glycine max* L. radicles exposed to various treatments of arsenic and/ or salicylic acid for two and five days. In this graph, C = control, T1 = 500 μ M SA, T2 = 10 μ M As, T3 = 10 μ M As + 500 μ M SA, T4 = 100 μ M As and T5 = 100 μ M As + 500 μ M SA. Each bar is mean $\pm$ SD of five distinct observations. Data were statistically analyzed separately for both the harvest days. Different lowercase letters indicate significant differences at $P < 0.05$.

Figure 2. Native-PAGE analyses of superoxide dismutase of *Glycine max* L. under treatments of arsenic alone or along with salicylic acid, on 2nd and 5th days of incubation. In this figure, C = control, T1 = 500 μ M SA, T2 = 10 μ M As, T3 = 10 μ M As + 500 μ M SA, T4 = 100 μ M As and T5 = 100 μ M As + 500 μ M SA.

Figure 3. Variation in the catalase activity of *Glycine max* L. radicles incubated in various concentrations of arsenic and/or salicylic acid for two and five days. In this graph, C = control, T1 = 500 μ M SA, T2 = 10 μ M As, T3 = 10 μ M As + 500 μ M SA, T4 = 100 μ M As and T5 = 100 μ M As + 500 μ M SA. Data of both the days were analyzed separately. Each bar is mean $\pm$ SD of five distinct observations. Different alphabets indicate significant differences at $P < 0.05$.

Figure 4. Isozyme pattern of catalase enzyme of *Glycine max* L. radicles exposed to arsenic and/or salicylic acid for two and five days. In this figure, C = control, T1 = 500 μ M SA, T2 = 10 μ M As, T3 = 10 μ M As + 500 μ M SA, T4 = 100 μ M As and T5 = 100 μ M As + 500 μ M SA.

Analysis of CAT in As-treated *Glycine max* L. revealed presence of two isoforms (Figure 4). These are relatively more intense in T2 (10 μ M As), T3 (10 μ M As + 500 μ M SA) and T5 (100 μ M As + 500 μ M SA) grown radicles, than the T4 (100 μ M As). Moreover, these are more intense in 5th day samples than the 2nd day, and are well supported by their spectrophotometric data (Figures 3 and 4).

Native-PAGE of POD resolved six isoforms of it (Figure 6). Relative to the control, intensities of isoforms-

to V were increased substantially with increased As, but restored with exogenous SA addition on both the days of analysis. Analysis of APX identified three and six isoforms on 2nd and 5th days respectively (Figure 8). Isoforms-III and VI is common in all the samples of both the days.

Additionally, two new isoforms (II and IV) were found to be induced exclusively in response to SA on 5th day of treatment, but were absent on 2nd day.

Figure 5. Change in the activity of guaiacol peroxidase in growing radicles of *Glycine max* L. under different concentrations of arsenic and/ or salicylic acid for two different time lengths. In this graph, C = control, T1 = 500 μ M SA, T2 = 10 μ M As, T3 = 10 μ M As + 500 μ M SA, T4 = 100 μ M As and T5 = 100 μ M As + 500 μ M SA. Each bar is mean $\pm$ SD of five distinct observations. Data of both the days were statistically analyzed separately. Different letters indicate significant differences at $P < 0.05$.

Figure 6. Isozymic analyses of guaiacol peroxidase enzyme of arsenic and/ or salicylic acid treated *Glycine max* L. radicles of 2nd and 5th days. In this figures, C = control, T1 = 500 μ M SA, T2 = 10 μ M As, T3 = 10 μ M As + 500 μ M SA, T4 = 100 μ M As and T5 = 100 μ M As + 500 μ M SA.

3.6. Proline

The level of proline in *Glycine max* L. significantly ($P < 0.05$) enhanced in response to As (Table 1). Proline accumulation was found to be higher (858%) in high As (T4: 100 μ M) subjected seedlings than that of low As (T2: 100 μ M). Data exhibited a close association of it with As concentration

($r = 0.98$, $P < 0.05$), and length of exposure ($r = 0.99$, $P < 0.05$). Gathered data revealed that exogenous SA (T3 and T5) stimulated accumulation of proline as compared with the corresponding As level (T2 and T4). Maximum amount of proline (2.66 mg g⁻¹ FM) was measured in T5 (100 μ M As + 500 μ M SA) subjected radicles (Table 1).

Figure 7. Activity of ascorbate peroxidase in *Glycine max* L. radicles exposed to various treatments of arsenic and/ or salicylic acid for two and five days. In this graph, C = control, T1 = 500 μ M SA, T2 = 10 μ M As, T3 = 10 μ M As + 500 μ M SA, T4 = 100 μ M As and T5 = 100 μ M As + 500 μ M SA. Data of both the days were separately analyzed. Each bar is mean $\pm$ SD of five distinct observations. Different small alphabets indicate significant differences at $P < 0.05$.

Figure 8. Native-PAGE analyses of ascorbate peroxidase of *Glycine max* L. seedlings exposed to various concentrations of arsenic and/ or salicylic acid for two and five days. In this figure, C = control, T1 = 500 μ M SA, T2 = 10 μ M As, T3 = 10 μ M As + 500 μ M SA, T4 = 100 μ M As and T5 = 100 μ M As + 500 μ M SA.

4. Discussion

Arsenic-toxicity constitutes one of the major abiotic threats that not only disturb crop production instead human health also. Recent reports revealed that exogenous application of certain chemicals may result in the alleviation of various abiotic stresses that could be important from both theoretical and applied point of views (Namdjoyan and Kermanian,

2013; Singh *et al.*, 2015a). Among such chemicals, SA is recently receiving increasing attention due to its involvement in the plant growth regulation and abiotic stress tolerance mechanism (Dong *et al.*, 2015). Inhibitory impact of As on *Glycine max* L. seed was scrutinized by recording the changes in germination rate, length and DM of the radicles under pre-fixed doses of As (10 and 100 μ M) and increase in exposure time (2 and 5 days). Study of

time-dependent alterations is a functional approach to assess time course changes in DM accumulation. In general, growth and DM accumulation of *Glycine max* L. were negatively ($r = -0.98$, $P < 0.05$) influenced by As (Table 1). This growth inhibition could partly be due to the reduction in cell division rate (Chandrarakar *et al.*, 2016). Our observations are substantiated with those of Chun-xi *et al.* (2007) and Singh *et al.* (2015b). Arsenic is well known to affect adversely the plants growth and development upon its accumulation. A study conducted on *Solanum melongena* L. also demonstrated such a concentration dependent influx of As (Singh *et al.*, 2015b). In the present study, exogenous SA significantly counteracted the reductions in growth caused due to As-toxicity by lowering its accumulation in *Glycine max* L. Moreover, SA was shown to involve actively in the regulation of overall growth and development of the plants (Kazemi *et al.*, 2010).

Contamination of As in plants has closely been linked to oxidative damage via over production of ROS. Of note, our results indicated that amount of ROS increased significantly (524-2880%) with As addition (Table 2), hence correlated with length of exposure ($r = 0.99$, $P < 0.05$) and As concentration ($r = 0.86$, $P < 0.05$). Like ours, As-induced rise in the ROS was also documented in the *Phaseolus aureus* L. and *Nasturtium officinale* (Kaur *et al.*, 2012; Namdjoyan and Kermanian, 2013). While exogenous SA counteracted the As-induced increase in ROS levels (Table 2). Our findings are in line with that of Odjegba (2012) and Singh *et al.* (2015a) in *Arabidopsis thaliana* and *Oryza sativa* L. SA mediated lowering in ROS indicates a protective role against As-stress.

Addition of As manifested remarkable rise (744%, $P < 0.05$) in the LOX activity as compared to control (Table 2). Similar change was also noted in As-stressed *Nasturtium officinale* (Namdjoyan and Kermanian, 2013). It has been proposed that stress-induced

increment in LOX is a reflection of higher lipolytic activity in cellular membranes, and increased oxidation of membrane-bound fatty acids (Kazemi *et al.*, 2010). However, in this study exogenous SA declined (~50%) the LOX activity in *Glycine max* L., and was well supported by the report of Kazemi *et al.* (2010). Alleviation of oxidative damage by scavenging ROS via antioxidant enzymes is an important strategy of plants to protect them against stresses. In this study, activities of SOD, CAT, POD and APX were altered in response to As and/ or SA (Figures 1-8). Similar change in these enzymes has also been observed by Chun-xi *et al.* (2007) and Kaur *et al.* (2012). However, exogenous SA restored the activities of tested enzymes thereby reduced ROS and oxidative damage in *Glycine max* L. Data approves that the key role of SA is to serve as a signaling molecule and thereby enhancing the defensive system. Similar results have also been observed by Singh *et al.* (2015a).

Isozyme charts are molecular level types after gene expression. The intensity of isoform can reflect relative activity of any enzyme. Native-PAGE revealed seven and two isoforms of SOD and CAT respectively in *Glycine max* L. subjected to As and/or SA (Figures 2 and 4). Similar patterns of these were also observed by Chun-xi *et al.* (2007). POD exists in a number of isoforms and has diverse functions in plants metabolism depending on their substrates. H_2O_2 has been reported to serve as an essential substrate of POD (Chun-xi *et al.*, 2007). Native-PAGE of POD resolved six bands with different intensities in response to As and SA (Figure 6). Compared to control, intensities of isozymes-I to V were increased in response to As, but restored with SA, approving its involvement in restoring POD activity in As-stressed *Glycine max* L. Our results are in coherence with Chun-xi *et al.* (2007). Native-PAGE of APX unveiled three and six isoforms on 2nd and 5th days respectively (Figure 8). Moreover, exogenous SA induced two new isoforms (II and IV)

on 5th day of treatment, but were absent on 2nd day. Mostofa and Fujita (2013) showed that activity of APX was enhanced after addition of SA into *Oryza sativa* L. Results suggests that exogenous SA regulates antioxidative enzymes, thereby lowers ROS and prevent *Glycine max* L. against As-stress.

Proline was shown to be accumulated in plants under abiotic stresses (Agami, 2014), and was also found true in this study (Table 1). These results are in agreement with that of Namdjoyan and Kermanian (2013) and Singh *et al.* (2015b). Its accumulation could possibly be the resultant of As-accrued water imbalance inside the cells. In fact, proline is not only an important molecule in redox signaling, but also serves as a potential ROS scavenger (Agami, 2014).

5. Conclusions

Accumulated data clearly demonstrate that interactive effects of As and SA considerably reduced As-toxicity in *Glycine max* L. (variety JS335). Arsenic inhibited radicle emergence and then elongation in germinating *Glycine max* L. seeds by inducing ROS-mediated oxidative damage and altering the activities of antioxidant enzymes. Exogenous SA could enhance tolerance of the *Glycine max* L. to the As-stress through up-regulating the activities of SOD, CAT and APX, and accumulation of proline. Therefore, it can be concluded that the supplementation of SA proved to be beneficial for the plants in combating As-stress. However, further investigation is needed, particularly at gene expression level, to gain deeper understanding in regard to the interaction of As and SA in plants.

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