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Exogenous salicylic acid alleviates arsenic toxicity in *Arabidopsis thaliana*

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Abstract

The present study investigated the possible protective role of salicylic acid (SA) against arsenic toxicity in *Arabidopsis thaliana*. Seedlings were raised from seeds in plastic containers filled with commercial propagation medium and were divided into two groups. Half of the seedlings were treated with 250 µM salicylic acid for 2 weeks, after which both groups were exposed to 100 µM arsenic for 2 weeks. Metabolic parameters representative of oxidative damage and antioxidant activity were evaluated after the treatments. The result showed that arsenic caused a decrease in plant biomass, chlorophyll content and a significant increase in lipid peroxidation, and activities of catalase, ascorbate peroxidase and superoxide dismutase in seedlings that were not pretreated with SA. The toxic effects of arsenic were however alleviated by the exogenously applied SA thereby underscoring the beneficial role of this signal molecule in mediating defense response in plants under stress.

Keywords: Antioxidant enzymes; Metals; Oxidative stress; Signal molecule; Pollution; Environment.

Abbreviations: AsV - Arsenate; AsIII - Arsenite; ATP – Adenosine tri phosphate; OH^- - hydroxyl radicals; O_2^- - Superoxide radicals; H_2O_2 - hydrogen peroxide; ROS - Reactive oxygen species; SOD - Superoxide Dismutase; POD – Peroxidase; GR - Glutathione Reductase; SA - Salicylic acid; MDA – Malondialdehyde; CAT – Catalase; APX - Ascorbate peroxidase; NBT - Nitro blue tetrazolium; SE - Standard error; ANOVA - One-way analysis of variance; LB - Luria-Bertani.

Introduction

Arsenic is a naturally occurring metalloid that is toxic to most organisms. It is found in soils and ground water in many regions of the world, and its presence in an elevated concentration is a significant environmental problem that has attracted the attentions of many researchers in the past decade. Arsenic exists in two main forms in the environment, as arsenate (AsV) or arsenite (AsIII), depending on the redox potential of the environment (Cullen & Reimer, 1989). Arsenate, a phosphate analog, is the predominant form of arsenic in aerated soils. When taken up by plants, it interferes with various metabolic pathways in cells like, interaction with sulfhydryl groups and replacement of phosphate from ATP. As a result, low biomass and crop yield are among the major responses of plants to arsenic pollution (Knauer *et al*., 1999). Heavy metals including metalloid arsenic have been reported to stimulate the formation of free radicals and reactive oxygen species leading to oxidative stress (Zhang *et al*., 2003).

It is well known that biotic and abiotic stresses disturb redox homeostasis in plant cells and induce the accumulation of reactive oxygen species (ROS) (Grant & Loake, 2000). ROS, including hydroxyl radicals (OH^-), superoxide radicals (O_2^-) and hydrogen peroxide (H_2O_2), can damage cell membranes, chloroplast pigments, lipids, nucleic acids and proteins (Tewari *et al*., 2002). To overcome the oxidative damage, plants have developed an extensive network of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), ascorbate peroxidase (APX), glutathione reductase (GR) and low molecular weight antioxidants, such as glutathione (Mittler, 2002). When plants are exposed to severe or prolonged stress conditions, the detoxification capacity of the antioxidant enzymes may not meet up with the rate at which the oxy-radicals are generated, hence, there is likelihood of oxidative damage under these conditions. Therefore, the search for chemical substances or molecules capable of inducing stress tolerance in plants is of

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importance in order to understand how plants tolerate adverse conditions. Various agents, including jasmonic acid, abscisic acid or salicylic acid, may mediate the acclimation of plants to environmental stress (Ding *et al*., 2002; Rao & Davis, 1999; Tsonev *et al*., 1998; Senaratna *et al*., 2000; Zeevaart & Creelmann, 1988) and interact with other cellular metabolites and environmental factors in the regulation of stress responses. Evidence indicates that salicylic acid (SA) is a natural signal molecule for the activation of plant defense responses (Klessig & Malamy, 1994).

Plant growth and development are hampered by various biotic and abiotic stress factors. Scientific evaluation of chemical substances or molecules capable of conferring stress tolerance qualities on sensitivity plant species are of great importance. Plant hormones play an important role in developmental processes, and some of them have key roles in mechanisms leading to acclimation to changing environments. Salicylic acid (SA) has been known as a signal molecule in the induction of defense mechanisms in plants (Raskin, 1992; Shah, 2003). Recent studies suggest that it also participates in signaling during abiotic stresses (Horvath *et al*., 2007). SA and certain related compounds protected maize plants against chilling injury (Janda *et al*., 1999), decreased the inhibitory effect of drought and salt stress in wheat (Al-Hakimi & Hamada, 2001), decreased the effect of paraquat on photosynthesis (Ananieva *et al*., 2002), were involved in the modulation of salt and osmotic stresses (Borsani *et al*., 2001) and induced multiple stress tolerance in bean and tomato plants (Senaratna *et al*., 2000). This study was undertaken to determine the physiological and biochemical changes in *Arabidopsis thaliana* plants treated with SA during arsenic-induced stress, and to investigate whether exogenous application of this plant regulator can induce tolerance to arsenic stress.

Materials and methods

Plant growth and treatment

The study was conducted in the Department of Horticultural Sciences, University of Florida, in November and December, 2010. Wild type, *Arabidopsis thaliana* L. ecotype Columbia were grown from seeds in plastic containers (6.5 x 6.5 x 6.0 cm) filled with a commercial propagation medium (Mix number 2, Fafard Inc., Agawam, MA, USA). The plants were grown under 16-h-light photoperiod of 150 µmol s^{-1} m^{-2} supplied by cool white fluorescent lights at 24/20°C day/night temperatures. After germination, thinning was done to achieve plant stand of one seedling per container. Plants were allowed to grow for 2 weeks and were then divided into two groups. One group received a daily supply of 30 ml 250 µM salicylic acid for 2 weeks while the other group of plants received equal volume of water devoid of SA for the same period. Plants in each of the groups were further categorized into two, so that some were exposed to a daily supply of 30 ml 100 µM arsenic (supplied as Na_2HAsO_4) for 2 weeks, while others were supplied water. The experimental set up was completely randomized and each treatment was replicated 10 times.

Dry weight determination

Harvested plants were washed thoroughly in a running tap water to remove soil particles. After rinsing with distilled water, they were placed in labeled paper bags and oven dried at 70°C for 72 hours. The dried samples were weighed using a digital top loading weighing balance (Mettler AE 100) to determine the dry weight.

Determination of total chlorophyll

Plant leaves (100 mg) were ground in 10ml 80% acetone in the dark. After centrifugation at 4000 g for 5 min, the absorbance of the supernatant was read at 645 and 663 nm (Arnon, 1949). The total chlorophyll content was calculated using the formula given by Maclachalan & Zalik, 1963).

Lipid peroxidation

Lipid peroxidation was measured by estimation of the malondialdehyde (MDA) content following a modified procedure of Wang & Jin (2005).

Enzyme determination

For enzyme analysis, fresh samples of leaves (200 mg each) were ground in a chilled mortar and extracted with 5 ml of ice-cold 100 mM potassium phosphate buffer pH 7.5, with 1% (w/v) polyvinyl

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pyrrolidone. The homogenate was centrifuged at 15,000 rpm for 10 min. The supernatant was used for the estimation of antioxidant enzyme activities.

Catalase (CAT) activity was determined according to Aebi (1984). Ascorbate peroxidase (APX) activity was assayed according to the method of (Nakano & Asada, 1981). The oxidation of ascorbate was determined by the change in absorbance at 290nm. SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT) according to the method of Beauchamp and Fridovich, 1971. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction at 560nm. Protein content was determined according to the method of Bradford (1976) with bovine serum albumin as a standard.

Salicylic acid extraction and detection

Salicylic acid was extracted from plant tissues according to the method of Marek *et al*. (2010). Uniform leaf discs (7.5 mm in diameter and 5mg by weight) were separately placed in PCR tubes containing 200 µl of Luria-Bertani (LB) and heated at 95^{o}C for 30 min in a PCR machine and cooled down to room temperature. An overnight culture of *Acinetobacter* sp. was diluted (1:20) in LB at 37^{o}C and grown for 2 hours at 200 rpm to an OD_{600} of 0.4. With the aid of multipipette, 50 µl of biosensor culture was added to each well of the cell culture plate, and then 50µl of the extract was added to each well and mixed thoroughly. The plate was incubated at 37^{o}C for 1 hr before luminescence was read using a Veritas Microplate Luminometer (Promega Corporation, Sunnyvale, California).

Determination of total arsenic

Freshly harvested whole plants were used for total arsenic extraction and quantification. Plants weighing 0.1g was extracted in 70% (v/v) methanol in water. Total arsenic was determined using an atomic absorption spectrophotometer (Varian 240Z, Zeeman Atomic Absorption Spectrophotometer, Varian, and Walnut Creek, CA, USA) by the method of Chen & Ma (1998).

Statistical analysis

All values reported in this study are the mean of three replicates. The data were expressed as mean values with standard error (SE). One-way analysis of variance (ANOVA) was used to assess the significance of the effects of the exogenous salicylic acid on the plant. Statistical analysis was performed with the SPSS 15.0 software package and *p* values <0.05 were considered significant.

Results

Effect of SA on growth response to arsenic toxicity

Whole plant dry weight as an index of growth was monitored in this study, and the result is as shown in Fig.1. Arsenic at the used dose caused a

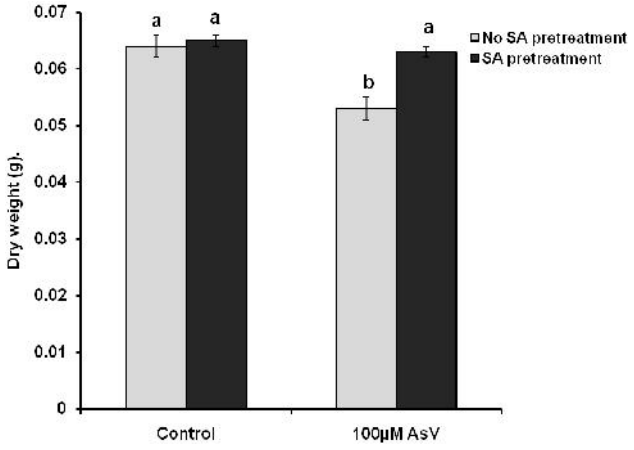

Fig.1. Whole plant dry weight of *A.thalina* as affected by arsenate ans SA treatments. Identical columns with the same letter are not significantly different at p<0.05

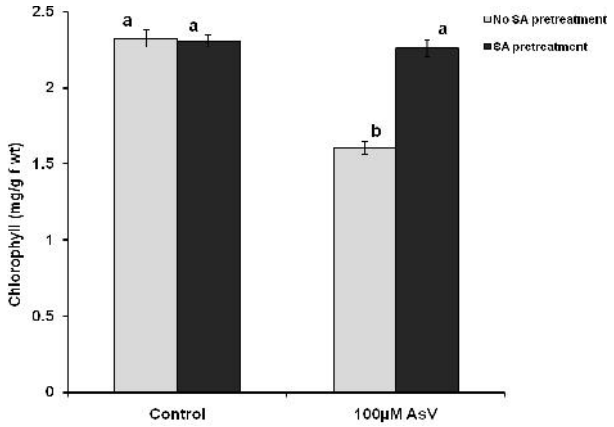

Fig.2. Effects of arsenate and SA on the chlorophyll content of *A. thalina*. Identical columns with same letter are not significantly different at p<0.05

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significant decrease in whole plant growth in the absence of SA pretreatment. This negative effect on growth was however not observed in plants pretreated with SA, as there was no significant difference in mean dry weight values of the control and those pretreated with SA before exposure to μM100 arsenic.

Effect on chlorophyll content

The chlorophyll content of *A. thaliana* exposed to arsenic with or without SA pretreatment is as presented in Fig.2. In the control plants, the total chlorophyll values recorded were 2.322 mg g^{-1} fwt and 2.306 mg g^{-1} f wt for plants without SA pretreatment and SA pretreated plants respectively. The level of chlorophyll was however reduced to 1.607 mg g^{-1} f wt when plants were exposed to arsenic without prior treatment with SA, while plants pretreated with SA before exposure to arsenic showed no significant difference in chlorophyll content compared to the control plants.

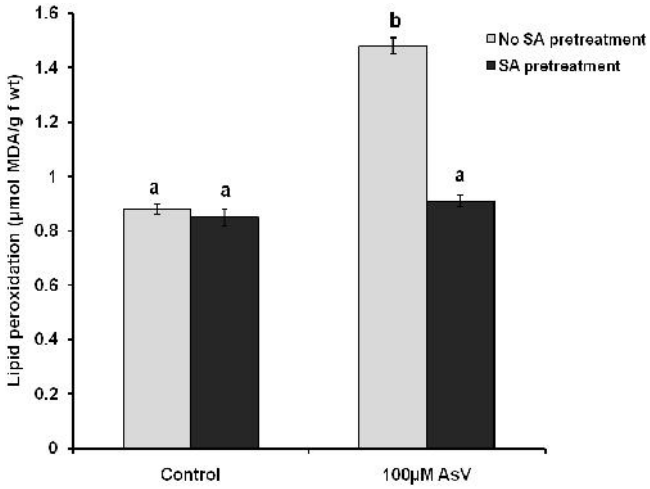

Fig. 3. MDA content of A.thaliana exposed to arsenate and SA. Identical columns with the same letter are not significantly different at p<0.05

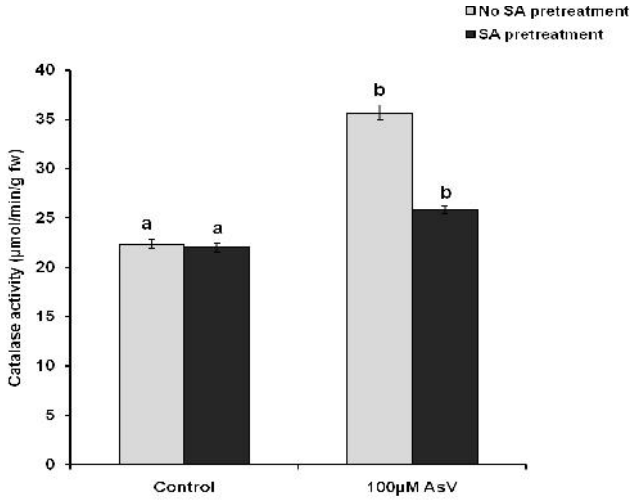

Fig.4. catalase activity in the leaves of *A.thalina* exposed to arsenate with or without SA pretreatment. Differentiation letters on identical columns are significanlty different at p<0.05

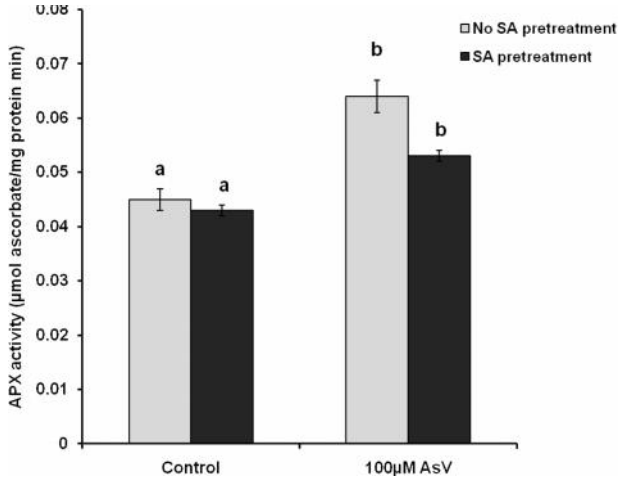

Fig.5. Ascorbate peroxidase activity in the leaves of *A.thalina* exposed to arsenate with or without SA pretreatment. Identical columns with different letters are significanlty different at p<0.05

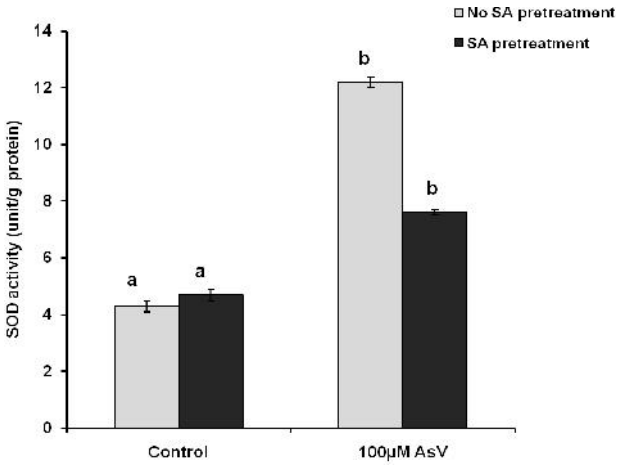

Fig.6. Superoxide dismutase activity in the leaves of *A. thalina* exposed to Arsenate with or without SA pretreatment. Identical columns with different letters are significantly different at p<0.05

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SA decreases arsenic toxicity-induced lipid peroxidation

Fig.3 compares MDA content of leaves from *A. thaliana* subjected to arsenic with or without SA pretreatment. MDA contents indicate lipid peroxidation and increased sharply upon exposure to arsenic without prior treatment with SA, but in plants pretreated with SA before exposure to arsenic exhibited low MDA content comparable to the control plants. Despite accumulation of similar amount of arsenic by these plants (with or without SA pretreatment) there was a significant difference in their MDA contents.

SA pretreatment lowered the arsenic-dependent increase in antioxidant enzymes

The activities of CAT, APX, and SOD were measured as representative enzymes involved in antioxidant metabolism, and the results are presented in Figs 4, 5, and 6 respectively. In arsenic exposed plants, there was a significant increase in the activities of CAT, APX, and SOD compared to the control plants. It was observed that the activities of the antioxidant enzymes in plants that were pretreated with SA before exposure to arsenic had lower levels of antioxidant activities than those without SA pretreatment. In the control plants, significant difference was not observed in the activity of the individual antioxidant enzymes, whether or not the plants were pretreated with SA. The activities of all the three antioxidant enzymes were observed to be at the maximum when plants were exposed to arsenic without prior treatment with SA.

SA pretreatment and arsenate increased free SA content in leaves of A. thaliana

In the control plants, exogenously applied SA led to a significant increase in the level of free SA in the leaves, indicating that the plants took up the applied SA. It was observed that exposure to 100 μM arsenic induced accumulation of SA in the leaves of plants grown with or without SA pretreatment (Fig.7). The increase in SA accumulation was more pronounced in plants exposed to arsenic without prior treatment with exogenous SA. It was observed in this study that SA pretreatment affected the arsenic-induced SA accumulation as plants exposed to arsenic with SA pretreatment had less amount of free SA compared to plants that were treated with arsenic without SA pretreatment (Fig.7).

SA pretreatment had no effect on arsenic accumulation in plants

In this study, it was observed that exogenously applied SA had no effect on the accumulation of arsenic by *A. thaliana*. As shown in Fig.8, there was no significant difference in the total arsenic

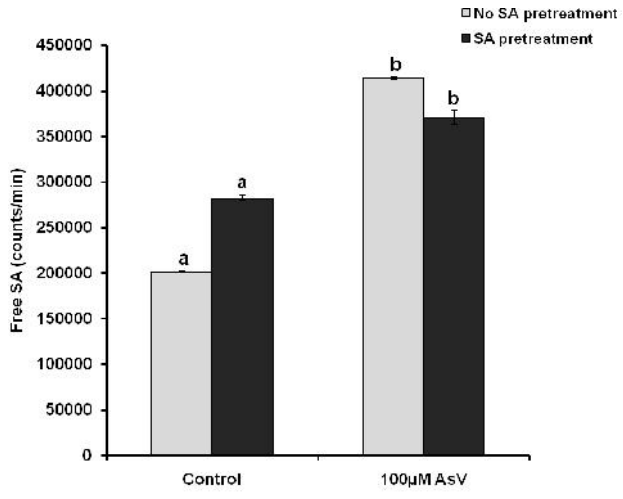

Fig.7. Free SA in the leaves of *A.thalina* exposed to arsenate with or without SA pretreatment. Different letters on similar columns indicates significant difference at $p<0.05$

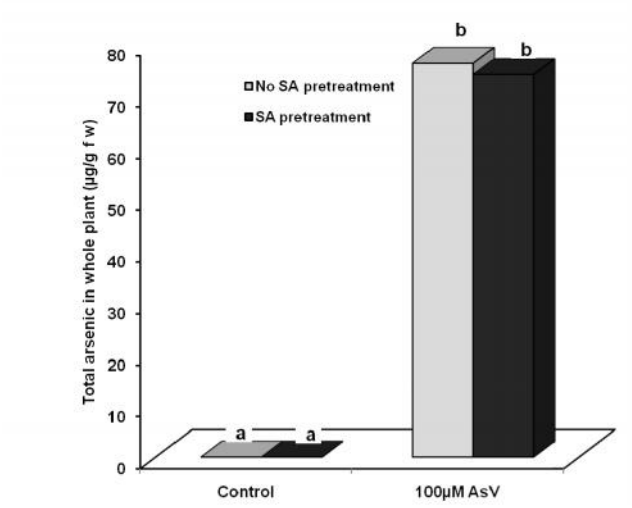

Fig.8. Total arsenic content in the root tissue of *A.thalina* with or without SA pretreatment. Different letters on identical columns are significantly different at $p<0.05$

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accumulated by plants with or without SA pretreatment. As expected, the control plants were devoid of arsenic, while plants without SA pretreatment and SA pretreated plants had mean values of 76.25 ± 0.39 and 74.06 ± 0.27 µg g^{-1} f.wt respectively.

Discussion

The present study was carried out to evaluate the beneficial effect of SA on *A. thaliana* plants exposed to toxic arsenic concentration. Arsenic toxicity has recently received increased attention due to its chronic effects on human health. The element has no known beneficial biological function, and its presence in the plant interferes with the metabolism and alters the uptake of other essential elements (Mallick, *et al.,* 2011), hence, the assessment of tolerance and sensitivity of plants to arsenic has become important because portions of land being contaminated by this toxic element is on the increase. Salicylic acid is known to affect various physiological and biochemical activities of plants and may play a key role in regulating their growth and productivity (Arberg, 1981).

In this work, it was observed that the negative effect of arsenic on the growth of *A. thaliana* as shown by the dry weight data was less severe in the SA pretreated plants, indicating that SA played a role in reducing the toxic effect of arsenic on the growth of the plant, and this agreed with the work of Shakirova (2007) where application of salicylic acid improved the growth of wheat exposed to salinity stress.

The data showed that the chlorophyll content was reduced in arsenic-treated plants that were not pretreated with SA (Fig.2). It was observed that exogenous application of SA prevented the negative impact of the arsenic on the photosynthetic pigment as the chlorophyll content was comparable to that the control. Similar result was reported by Ghai *et al*. (2002) when *Brassica napus* was sprayed with SA.

The MDA assay can be regarded as a reliable method for evaluating the level of lipid peroxidation in a biological system, and is being used as a toxicity bioassay for plants. An increase in the level of MDA indicates enhanced lipid peroxidation which results to poor structural integrity of the membrane. It is suggested that the inhibition of key enzyme systems, together with electron leakage during the conversion of arsenate to arsenite, leads to formation of reactive oxygen species (ROS), which in turn causes lipid peroxidation (Zhao *et al*., 2009). In this study, the MDA content of arsenic-treated plants that were not pretreated with exogenous SA was significantly higher than the values recorded for the control and arsenic-treated plants pretreated with SA. The result is an indication that arsenic-treated plants without prior exposure to exogenous SA experienced higher oxidative stress than the control as well as those pretreated with SA. This agreed with the result reported by Krantev *et al.* (2008) that SA pretreated maize plants had lower level of MDA when exposed to toxic dose of cadmium.

The induction of the activities of a group of enzymes is considered to play an important role in the cellular defense strategy against oxidative stress. SOD converts O_2^- (superoxide radical) to H_2O_2, acting as the first line defense against oxyradical-mediated stress (Mittler, 2002). APX is an important peroxidase enzyme in H_2O_2 detoxification as it is present in the cytosol, catalyzing the reduction of H_2O_2 to water using ascorbate as a reductant. CAT is also H_2O_2 reducing enzyme. Activities of all these three enzymes were found to be higher in arsenic-treated plants devoid of exogenous SA, indicating that scavenging of ROS generated by arsenic toxicity was performed by the three enzymes. The data suggest that SA plays an important role in protecting *Arabidopsis* plants from oxidative stress. It has been reported that exogenously applied SA enhanced the efficiency of antioxidant system in plants (Knorzer *et al*., 1999). Ananieva *et al*. (2004) also reported that exogenous SA leads to increased antioxidant capacity in leaves of barley exposed to paraquat.

In the present experiment, it was evident that *A. thaliana* took up the exogenously applied SA even though it was applied via the roots. Comparing the two sets of the control plants in relation to

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extractable free SA, the set that received SA exogenously had an increased level of free SA, obviously, the differentials was as a result of the exogenously applied SA. Similar observation has been reported by Krantev *et al*. (2008). The finding that arsenic induced an increase in SA contents underlined the potential significance of this phenolic compound for plant growth under stress conditions. It has been reported that the level of endogenous SA increased when plants were exposed to different stress factors such as mercury (Zhou et al., 2009), cadmium (Pal *et al*., 2002), pathogens (Smith *et al*., 1991), salinity (Borsani *et al*., 2001), temperature (Dat *et al*., 1998) and drought (Singh & Usha, 2003). The high levels of endogenous SA in *A. thaliana* induced by arsenic may likely play a key role in mediating defense response against arsenic toxicity.

It was shown in this study that exogenously applied SA did not affect arsenic uptake by *A. thaliana* suggesting that different transporters are involved in their uptake. It was observed that relatively the same quantity of arsenic was taken up by *A. thaliana* plants whether they were pretreated with SA or not, this further confirmed the beneficial role of SA, as exogenously applied SA reduced the toxic effects of arsenic in all the parameters evaluated in this study.

Conclusion

Based on the present study, it was found that arsenic caused a decrease in plant biomass, chlorophyll content and a significant increase in lipid peroxidation, and activities of CAT, APX and SOD, as a result of oxidative stress. However, the beneficial effect of SA in mediating defense response in plants under stressful condition was emphasized. It was shown that the toxic effects of arsenic on *A. thaliana* were alleviated by exogenous application of SA prior to arsenic treatment.

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